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amendments.*(54) Title: IMPROVED SACCHARIFICATION OF CELLULOSE BY CLONING AND AMPLIFICATION OF THE  $\beta$ -  
GLUCOSIDASE GENE OF TRICHODERMA RESEI

## (57) Abstract

A process for expressing extracellular  $\beta$ -glucosidase in a filamentous fungus by expressing a fungal DNA sequence encoding enhanced, deleted or altered  $\beta$ -glucosidase in a recombinant host microorganism is disclosed. Recombinant fungal cellulase compositions containing enhanced, deleted or altered expression of  $\beta$ -glucosidase is also disclosed.

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IMPROVED SACCHARIFICATION OF CELLULOSE BY CLONING  
AND AMPLIFICATION OF THE  $\beta$ -GLUCOSIDASE GENE OF  
TRICHODERMA REESEI

BACKGROUND OF THE INVENTION

5     1.   Field of the Invention.

          The present invention relates to cellulase  
preparations and compositions having increased or  
decreased cellulolytic capacity. The invention further  
relates to a nucleotide sequence of the bgl1 gene  
10    encoding extracellular  $\beta$ -glucosidase from a filamentous  
fungi, a plasmid vector containing the gene encoding  
extracellular  $\beta$ -glucosidase and transformant strains  
with increased copy numbers of the  $\beta$ -glucosidase (bgl1)  
gene introduced into the genome. More particularly,  
15    the present invention relates to Trichoderma reesei  
strains that have increased or no levels of expression  
of the bgl1 gene resulting in enhanced or no  
extracellular  $\beta$ -glucosidase protein levels that can be  
used in conjunction with other compositions to produce  
20    a cellulase product having increased or decreased  
cellulolytic capacity.

2.   State of the Art.

          Cellulases are known in the art as enzymes that  
hydrolyze cellulose ( $\beta$ -1,4-glucan linkages), thereby  
25    resulting in the formation of glucose, cellobiose,  
cellooligosaccharides, and the like. As noted by Wood  
et al., "Methods in Enzymology", 160, 25, pages 234 et  
seq. (1988) and elsewhere, cellulase produced by a  
given microorganism is comprised of several different

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enzyme classes including those identified as exocellobiohydrolases (EC 3.2.1.91) ("CBH"), endoglucanases (EC 3.2.1.4) ("EG"),  $\beta$ -glucosidases (EC 3.2.1.21) ("BG"). Moreover, the fungal classifications of CBH, EG and BG can be further expanded to include multiple components within each classification. For example, multiple CBHs and EGs have been isolated from a variety of bacterial and fungal sources including Trichoderma reesei which contains 2 CBHs, i.e., CBH I and CBH II, and at least 3 EGs, i.e., EG I, EG II, and EG III components.

The complete cellulase system comprising components from each of the CBH, EG, and BG classifications is required to efficiently convert crystalline forms of cellulose to glucose. Isolated components are far less effective, if at all, in hydrolyzing crystalline cellulose. Moreover, a synergistic relationship is observed between the cellulase components particularly if they are of different classifications. That is to say, the effectiveness of the complete cellulase system is significantly greater than the sum of the contributions from the isolated components of the same classification. In this regard, it is known in the art that the EG components and CBH components synergistically interact to more efficiently degrade cellulose. See, for example, Wood, Biochem. Soc. Trans., 13, pp. 407-410 (1985).

The substrate specificity and mode of action of the different cellulase components varies with classification, which may account for the synergy of the combined components. For example, the current accepted mode of cellulase action is that endoglucanase



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components hydrolyze internal  $\beta$ -1,4-glucosidic bonds, particularly, in regions of low crystallinity of the cellulose and exo-cellobiohydrolase components hydrolyze cellobiose from the non-reducing end of  
5 cellulose. The action of endoglucanase components greatly facilitates the action of exo-cellobiohydrolases by creating new chain ends which are recognized by exo-cellobiohydrolase components.

$\beta$ -Glucosidases are essential components of the  
10 cellulase system and are important in the complete enzymatic breakdown of cellulose to glucose. The  $\beta$ -glucosidase enzymes can catalyze the hydrolysis of alkyl and/or aryl  $\beta$ -D-glucosides such as methyl  $\beta$ -D-glucoside and p-nitrophenyl glucoside, as well as  
15 glycosides containing only carbohydrate residues, such as cellobiose. The catalysis of cellobiose by  $\beta$ -glucosidase is important because it produces glucose for the microorganism and further because the accumulation of cellobiose inhibits cellobiohydrolases and endoglucanases thus reducing the rate of hydrolysis  
20 of cellulose to glucose.

Since  $\beta$ -glucosidases can catalyze the hydrolysis of a number of different substrates, the use of this enzyme in a variety of different applications is  
25 possible. For instance, some  $\beta$ -glucosidases can be used to liberate aroma in fruit by catalyzing various glucosides present therein. Similarly, some  $\beta$ -glucosidases can hydrolyze grape monoterpenyl  $\beta$ -glucosidase which upon hydrolysis, represents an  
30 important potential source of aroma to wine as described by Günata et al, "Hydrolysis of Grape

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Monoterpenyl  $\beta$ -D-Glucosides by Various  $\beta$ -Glucosidases",  
J. Agric. Food Chem., Vol. 38, pp. 1232-1236 (1990).

Furthermore, cellulases can be used in conjunction  
with yeasts to degrade biomass to ethanol wherein the  
cellulose degrades cellobiose to glucose that yeasts  
can further ferment into ethanol. This production of  
ethanol from readily available sources of cellulose can  
provide a stable, renewable fuel source. The use of  
ethanol as a fuel has many advantages compared to  
petroleum fuel products such as a reduction in urban  
air pollution, smog, and ozone levels, thus enhancing  
the environment. Moreover, ethanol as a fuel source  
would reduce the reliance on foreign oil imports and  
petrochemical supplies.

But the major rate limiting step to ethanol  
production from biomass is the insufficient amount of  
 $\beta$ -glucosidase in the system to efficiently convert  
cellobiose to glucose. Therefore, a cellulase  
composition that contains an enhanced amount of  
 $\beta$ -glucosidase would be useful in ethanol production.

Contrarily, in some cases, it is desirable to  
produce a cellulase composition which is deficient in,  
and preferably free of  $\beta$ -glucosidase. Such compositions  
would be advantageous in the production of cellobiose  
and other cellooligosaccharides.

$\beta$ -glucosidases are present in a variety of  
prokaryotic organisms, as well as eukaryotic organisms.  
The gene encoding  $\beta$ -glucosidase has been cloned from  
several prokaryotic organisms and the gene is able to  
direct the synthesis of detectable amounts of protein  
in E. coli without requiring extensive genetic

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engineering, although, in some cases, coupling with a promotor provided by the vector is required. However,  $\beta$ -glucosidases are not produced by such organisms in commercially feasible amounts.

5 Furthermore, such prokaryotic genes often cannot be expressed and detected after transformation of the eukaryotic host. Thus, in order to use fungal strains, fungal genes would have to be cloned using methods described herein or by detection with the T. reesei  
10 bgl1 gene by nucleic acid hybridization.

The contribution and biochemistry of the  $\beta$ -glucosidase component in cellulose hydrolysis is complicated by the apparent multiplicity of enzyme forms associated with T. reesei and other fungal  
15 sources (Enari et al, "Purification of Trichoderma reesei and Aspergillus niger  $\beta$ -glucosidase", J. Appl. Biochem., Vol. 3, pp. 157-163 (1981); Umile et al, "A constitutive, plasma membrane bound  $\beta$ -glucosidase in Trichoderma reesei", FEMS Microbiology Letters, Vol.  
20 34, pp. 291-295 (1986); Jackson et al, "Purification and partial characterization of an extracellular  $\beta$ -glucosidase of Trichoderma reesei using cathodic run, polyacrylamide gel electrophoresis", Biotechnol. Bioeng., Vol. 32, pp. 903-909 (1988)). These and many  
25 other authors report  $\beta$ -glucosidase enzymes ranging in size from 70-80 Kd and in pI from 7.5-8.5. More recent data suggests that the extracellular and cell wall associated forms of  $\beta$ -glucosidase are the same enzyme (Hofer et al, "A monoclonal antibody against the  
30 alkaline extracellular  $\beta$ -glucosidase from Trichoderma reesei: reactivity with other Trichoderma  $\beta$ -glucosi-

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dases", Biochim. Biophys. Acta, Vol. 992, pp. 298-306 (1989); Messner and Kubicek, "Evidence for a single, specific  $\beta$ -glucosidase in cell walls from Trichoderma reesei QM9414", Enzyme Microb. Technol., Vol. 12, pp. 685-690 (1990)) and that the variation in size and pI is a result of post translational modification and heterogeneous methods of enzyme purification. It is unknown whether the intracellular  $\beta$ -glucosidase species with a pI of 4.4 and an apparent molecular weight of 98,000 is a novel  $\beta$ -glucosidase (Inglin et al, "Partial purification and characterization of a new intracellular  $\beta$ -glucosidase of Trichoderma reesei", Biochem. J., Vol. 185, pp. 515-519 (1980)) or a proteolytic fragment of the alkaline extracellular  $\beta$ -glucosidase associated with another protein (Hofer et al, supra).

Since a major part of the detectable  $\beta$ -glucosidase activity remains bound to the cell wall (Kubicek, "Release of carboxymethylcellulase and  $\beta$ -glucosidase from cell walls of Trichoderma reesei", Eur. J. Appl. Biotechnol., Vol. 13, pp. 226-231 (1981); Messner and Kubicek, supra; Messner et al, "Isolation of a  $\beta$ -glucosidase binding and activating polysaccharide from cell walls of Trichoderma reesei", Arch. Microbiol., Vol. 154, pp. 150-155 (1990)), commercial preparations of cellulase are thought to be reduced in their ability to produce glucose because of relatively low concentrations of  $\beta$ -glucosidase in the purified cellulase preparation.

To overcome the problem of  $\beta$ -glucosidase being rate limiting in the production of glucose from cellulose

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using cellulase produced by a filamentous fungi, the art discloses supplementation of the cellulolytic system of Trichoderma reesei with the  $\beta$ -glucosidase of Aspergillus and the results indicate an increase in rate of saccharification of cellulose to glucose. Duff, Biotechnol Letters, 7, 185 (1985). Culturing conditions of the fungi have also been altered to increase  $\beta$ -glucosidase activity in Trichoderma reesei as illustrated in Sternberg et al, Can. J. Microbiol., 23, 139 (1977) and Tangnu et al, Biotechnol. Bioeng., 23, 1837 (1981), and mutant strains obtained by ultraviolet mutation have been reported to enhance the production of  $\beta$ -glucosidase in Trichoderma reesei. Although these aforementioned methods increase the amount of  $\beta$ -glucosidase in Trichoderma reesei, the methods lack practicality and, in many instances, are not commercially feasible.

A genetically engineered strain of Trichoderma reesei or other filamentous fungi that produces an increased amount of  $\beta$ -glucosidase would be ideal, not only to produce an efficient cellulase system, but to further use the increased levels of expression of the bgl1 gene to produce a cellulase product that has increased cellulolytic capacity. Such a strain can be feasibly produced using transformation.

But, in order to transform mutant strains of Trichoderma reesei or other filamentous fungi, the amino acid sequence of the bgl1 gene of Trichoderma reesei or the other filamentous fungi must be first characterized so that the bgl1 gene can be cloned and

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introduced into mutant strains of Trichoderma reesei or other filamentous fungi.

5 Additionally, once the bgl1 gene has been identified, information within linear fragments of the bgl1 gene can be used to prepare strains of Trichoderma reesei and other filamentous fungi which produce cellulase compositions free of  $\beta$ -glucosidase.

10 Accordingly, this invention is directed, in part, to the characterization of the bgl1 gene that encodes for extracellular or cell wall bound  $\beta$ -glucosidase from Trichoderma reesei and other filamentous fungi. This invention is further directed to the cloning of the bgl1 gene into a plasmid vector that can be used in the transformation process, and to introduce the bgl1 gene  
15 into the Trichoderma reesei or other filamentous fungi genome in multiple copies, thereby generating transformed strains which produce a cellulase composition having a significant increase in  $\beta$ -glucosidase activity. Moreover, cellulase compositions that  
20 contain increased cellulolytic capacity are also disclosed.

This invention is further directed, in part, to the deletion or disruption of the bgl1 gene from the Trichoderma reesei or other filamentous fungi genome.  
25 In addition, altered copies of the bgl1 gene which may change the properties of the enzyme can be reintroduced back into the Trichoderma reesei or other filamentous fungi genome.

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## SUMMARY OF THE INVENTION

The amino acid sequence of the extracellular or cell wall bound  $\beta$ -glucosidase protein from Trichoderma reesei has now been obtained in sufficient detail to  
5 enable the bgl1 gene to be cloned into a suitable plasmid vector. The plasmid vector can then be used to transform strains of filamentous fungi to produce transformants which have multiple copies of the bgl1 gene introduced therein.

10 Accordingly, in one of its process aspects, the present invention relates to a process for expressing enhanced extracellular  $\beta$ -glucosidase in a filamentous fungus comprising expressing a fungal DNA sequence encoding enhanced  $\beta$ -glucosidase in a recombinant host  
15 microorganism, said recombinant host microorganism being a filamentous fungus transformed with an expression vector containing said DNA sequence.

In another process aspect, the present invention relates to a process for expressing cellulases from a  
20  $\beta$ -glucosidic filamentous fungi which are free of extracellular  $\beta$ -glucosidase.

In yet another process aspect, the present invention relates to a process for expressing an altered extracellular  $\beta$ -glucosidase in a filamentous  
25 fungus.

In another aspect, the present invention is directed to the amino acid sequence of extracellular  $\beta$ -glucosidase from Trichoderma reesei.

30 In yet another aspect, the present invention is directed to use of a nucleic acid fragment comprising the entire or partial nucleotide sequence of the T.

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reesei extracellular  $\beta$ -glucosidase gene as a probe to identify and clone out the equivalent bgl1 gene from other  $\beta$ -glucosidic filamentous fungi.

5 In one of its composition aspects, the present invention is directed to novel and useful transformants of Trichoderma reesei, which can be used to produce fungal cellulase compositions, especially fungal cellulase compositions enriched in  $\beta$ -glucosidase or  
10 deleted of  $\beta$ -glucosidase. Also contemplated in the present invention is the alteration of the bgl1 gene and the introduction of the altered bgl1 gene into T. reesei to produce transformants which can also be used to produce altered fungal cellulase compositions.

15 In another composition aspect, the present invention is directed to fungal cellulase compositions prepared via the transformed Trichoderma reesei strains.

#### BRIEF DESCRIPTION OF THE DRAWINGS

20 Fig. 1 is the nucleotide sequence and deduced primary amino acid structure of the entire T. reesei bgl1 gene.

Fig. 2 is a schematic representation of the vector pSASB-glu.

25 Fig. 3A is a figurative representation of the vector pSASABGlu bal pyr ( $\Delta 36$ ).

Fig. 3B is a figurative representation of the vector pUCAB-Glu A/R pyr ( $\Delta 12$ ).

Fig. 4 represents a Northern blot of total RNA isolated from the transformed strains of Trichoderma



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reesei following induction with sophorose using the probes of cbh2 and a 700 bp fragment of bgl1 cDNA.

Fig. 5A represents an autoradiograph of a Southern blot of T. reesei DNA illustrating the presence of  $\beta$ -glucosidase gene in wild type T. reesei (RL-P37) compared to strains of T. reesei genetically modified so as to not include the  $\beta$ -glucosidase gene ( $\Delta 12$  and  $\Delta 36$ ).

Fig. 5B represents an autoradiograph of a Northern blot of T. reesei RNA illustrating the expression of  $\beta$ -glucosidase gene in wild type T. reesei (RL-P37) compared to strains of T. reesei genetically modified so as to not include the  $\beta$ -glucosidase gene ( $\Delta 12$  and  $\Delta 36$ ).

Fig. 5C represents an analysis of the proteins expressed by P37 (wild type),  $\Delta 12$ , and  $\Delta 36$  strains of Trichoderma reesei and illustrates the absence of  $\beta$ -glucosidase in the proteins expressed by  $\Delta 12$  and  $\Delta 36$  strains Trichoderma reesei.

Fig. 6 represents an autoradiograph of Hind III digested genomic DNA from a T. reesei overproducing strain (lane 9) and transformants of pSASB-Glu (lanes 1-8), blotted and probed with the 700 bp  $\beta$ -Glu probe.

Fig. 7 represents a curve illustrating Avicel hydrolysis using the dosage, substrate:enzyme of 80:1 from an enriched recombinant  $\beta$ -glucosidase composition produced by the present invention.

Fig. 8 represents a curve illustrating PSC hydrolysis using the dosage, substrate:enzyme of 300:1 from an enriched recombinant  $\beta$ -glucosidase composition produced by the present invention.

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Fig. 9 represents a curve illustrating the rate of hydrolysis of a cellulosic diaper derived fibers using an enriched recombinant  $\beta$ -glucosidase composition produced by the present invention.

5 Figs. 10A and 10B are autoradiographs of Aspergillus nidulans, Neurospora crassa, Humicola grisea genomic DNA digested with Hind III and Eco RI, blotted and probed with a DNA fragment containing the bgl1 gene of Trichoderma reesei.

10 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS  
OF THE INVENTION

As used herein, the term "enhanced extracellular  $\beta$ -glucosidase" or "enhanced  $\beta$ -glucosidase" means that at least one additional copy of a gene encoding for  
15 extracellular  $\beta$ -glucosidase has been introduced into the genome.

The term "devoid of the bgl1 gene" means either that the bgl1 gene has been deleted from the genome and therefore cannot be expressed by the recombinant host  
20 microorganism; or that the bgl1 gene has been disrupted in the genome so that a functional extracellular  $\beta$ -glucosidase enzyme cannot be produced by the recombinant host microorganism.

The term "altered  $\beta$ -glucosidase" or "altered  
25  $\beta$ -glucosidase gene" means that the amino acid sequence of the expressed protein has been altered by removing, adding, and/or manipulating the nucleic acid sequence of the gene or the amino acid sequence of the protein.

The term "by recombinant means" denotes that a  
30 microorganism has been transformed with a DNA molecule

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created in a test-tube by ligating together pieces of DNA that are not normally contiguous.

The term "cellulase free of extracellular  $\beta$ -glucosidase" refers to a cellulase composition which does not contain functional extracellular  $\beta$ -glucosidase enzyme. Such compositions are preferably prepared by culturing a filamentous fungi wherein the  $\beta$ -glucosidase gene has been either deleted or disrupted. Preferably, these compositions are prepared by culturing a filamentous fungi wherein the  $\beta$ -glucosidase gene has been deleted.

The term "filamentous fungi" means any and all art recognized filamentous fungi.

The term " $\beta$ -glucosidic filamentous fungi" refers to those filamentous fungi which produce a cellulase composition containing  $\beta$ -glucosidase.

The term "cellooligosaccharide" refers to those oligosaccharide groups containing from 2-8 glucose units having  $\beta$ -1,4 linkages. Such cellooligosaccharides include cellobiose (diglucose having a  $\beta$ -1,4- linkage) and are preferably derived from cellulose.

More specifically, the present invention relates to the isolation and characterization of the bgl1 gene coding for the extracellular or cell wall bound protein from Trichoderma reesei (sometimes referred to as "T. reesei") and the specific nucleotide and amino acid sequence of this gene. The bgl1 gene is cloned into plasmid vectors, which are further used to produce transformed strains of T. reesei and other filamentous fungi having extra copies of the bgl1 gene inserted therein. These transformants are then used to produce

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cellulase compositions having increased  $\beta$ -glucosidase activity and thus enhanced cellulolytic degradation.

Besides enhancing cellulolytic degradation by inserting extra copies of the bgl1 gene into T. reesei strains, it is also contemplated by the present invention to produce transformed strains that are completely devoid of the bgl1 gene.

Also contemplated by the present invention is the manipulation of the amino acid sequence in the bgl1 gene itself. Alteration of the active sites on this enzyme may lead to a variety of different changes in catalytic conversion. For example, since  $\beta$ -glucosidase has both hydrolase and transferase activity, alteration of the amino acid sequence may result in the removal of hydrolase activity and an increase in transferase activity and, thus, facilitate the synthesis of  $\beta$  1-4 oligo-dextrins. Moreover, manipulation of the amino acid sequence of  $\beta$ -glucosidase may result in further changes in the system, such as different pH optima, different temperature optima, altered catalytic turn over rate ( $V_{max}$ ), altered affinity ( $K_m$ ) for cellobiose leading to an increased affinity for cellobiose or a decreased affinity for cellobiose resulting in a slower or zero rate of reaction, altered product inhibition profile such that lower or higher levels of glucose will inhibit  $\beta$ -glucosidase activity, and the like.

Moreover, a nucleic acid fragment containing the entire nucleotide sequence of the extracellular  $\beta$ -glucosidase gene in T. reesei or a portion thereof can also be labeled and used as a probe to identify and

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clone out the equivalent bgl1 gene in other filamentous fungi.

Generally, the present invention involves the isolation of the bgl1 gene from T. reesei by  
5 identifying a 700 bp cDNA fragment of the gene which is then used as a probe to identify a single T. reesei fragment containing the bgl1 gene which was subsequently cloned. Because of the species homology of the bgl1 gene, a probe employing a fragment of the  
10 bgl1 gene of T. reesei can be employed to identify the bgl1 gene in other cellulolytic microorganisms and, it is understood that the following description for T. reesei could also be applied to other  $\beta$ -glucosidic filamentous fungi.

15 In the case of T. reesei, this 6.0 kb fragment is then cloned into a pUC plasmid and a series of mapping experiments are performed to confirm that the entire bgl1 gene is contained in this fragment. The nucleotide sequence is then determined on both strands and the  
20 position of two introns can be confirmed by sequence analysis of bgl1 cDNA subclones spanning the intron/exon boundaries. After isolation of the bgl1 gene, additional bgl1 gene copies are then introduced into T. reesei or other filamentous fungal strains to  
25 increase the expression of  $\beta$ -glucosidase.

In contrast, the entire bgl1 gene can also be deleted from the genome of T. reesei and other  
30  $\beta$ -glucosidic filamentous fungi, thereby producing transformants that express cellulases free of  $\beta$ -glucosidase.

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The isolation of the bgl1 gene from T. reesei involves the purification of extracellular  $\beta$ -glucosidase, chemical and proteolytic degradation of this protein, isolation and determination of the sequence of the proteolytic fragments and design of synthetic oligomer DNA probes using the protein sequence. The oligomeric probes are then further used to identify a 700 bp  $\beta$ -glucosidase cDNA fragment which can be labeled and employed to later identify a fragment that contains the entire bgl1 gene within the fragment from digested genomic DNA from T. reesei.

To identify a feasible cDNA fragment that can be used as a probe for future analysis, total RNA is first isolated from T. reesei mycelia and polyadenylated RNA isolated therefrom. The polyadenylated RNA is then used to produce a cDNA pool which is then amplified using specific oligonucleotide primers that amplify only the specific cDNA fragment encoding the T. reesei bgl1 gene.

More specifically, total RNA is first isolated from a starting strain of T. reesei. The starting strain employed in the present invention can be any T. reesei cellulase overproduction strain that is known in the art. This cellulase producing strain is generally developed by ordinary mutagenesis and selection methods known in the art from any T. reesei strain. Confirmation that the selected strain overproduces cellulases can be performed by using known analysis methods. A preferred strain is RLP37 which is readily accessible.

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A mycelial inoculum from the T. reesei over production strain, grown in an appropriate growth medium, is added to a basal medium and incubated for a period of between 50-65 hours at a temperature between 25°C to 32°C, preferably 30°C. Fresh basal medium can be replaced during this incubation period. The culture medium is then centrifuged, and the mycelia is isolated therefrom and washed. The mycelia is then resuspended in a buffer to permit growth thereof and 1 mM sophorose (a  $\beta$ ,1-2 dimer of glucose) is added to the mycelia to induce the production of cellulase enzymes. The mycelia preparation is then incubated for an additional time period, preferably 18 hours at 30°C prior to harvesting.

Total RNA can be isolated from the mycelia preparation by a variety of methods known in the art, such as proteinase K lysis, followed by phenol:chloroform extraction, guanidinium isothiocyanate extraction, followed by cesium chloride gradients, guanidine hydrochloride and organic solvent extraction, and the like. It is preferable to isolate total RNA via the procedure described by Timberlake et al in "Organization of a Gene Cluster Expressed Specifically in the Asexual Spores of A. nidulans," Cell, 26, pp. 29-37 (1981). The mycelia is isolated from the culture medium via filtration. Then the RNA is extracted from the mycelia by the addition of an extraction buffer, TE-saturated phenol and chloroform. The aqueous phase is removed and the organic phase is reextracted with the extraction buffer alone by heating the extraction mixture in a water bath at a temperature

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between about 60°C to 80°C, preferably 68°C to release the RNA trapped in polysomes and at the interface. All of the extracted aqueous phases are then pooled, centrifuged and reextracted with phenol-chloroform until there is no longer any protein at the interface. The RNA is further precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol and pelleted via centrifugation before it is resuspended in DEP-water containing an RNase inhibitor.

10       The total RNA is then fractionated on 1% formaldehyde-agarose gels, blotted to Nytran™ membranes, and probed using a fragment of the T. reesei cbh2 gene to determine whether the genes encoding the enzymes of the cellulase system in the T. reesei preparation are indeed induced by addition of the sophorose. Basically, the probe used in the present invention is derived from a CBH II clone produced by methods known in the art. For more specific detail of how the clone was produced see Chen et al, "Nucleotide Sequence and Deduced Primary Structure of Cellobiohydrolase II from Trichoderma reesei," Bio/Technology, Vol. 5 (March 1987). Site directed mutagenesis was performed on the CBH II clone and a Bgl II site was placed at the exact 5' end of the open reading frame and a Nhe I site at the exact 3' end. The Bgl II and Nhe I restriction fragment containing CBH II coding sequence was further cloned into a pUC218 phagemid. The CBH II gene was further cut and gel isolated prior to adding a label.

30       The results of the Northern blot of T. reesei RNA probed with the cbh2 probe indicated that the level of



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cbh2 specific mRNA reached a peak at 14-18 hours post induction. From this data it can be inferred that the entire cellulase complex including  $\beta$ -glucosidase is induced at this time. The total RNA from 14, 18 and 22 hours is then pooled.

After pooling the specific fractions of total RNA, polyadenylated mRNA is further isolated from the total RNA. Postranscriptional polyadenylation is a common feature of the biogenesis of most eukaryotic mRNAs. The newly synthesized mRNAs have long poly(A) tracts which tend to shorten as mRNAs age. The newly synthesized polyadenylated mRNA is further isolated from total RNA by methods known in the art. These methods include the use of oligo(dT)-cellulose, poly(U) Sepharose, adsorption to and elution from poly(U) filters or nitrocellulose membrane filters, and the like. It is preferable to use oligo(dT) cellulose chromatography in isolating mRNA following the procedure described by Sambrook et al, Molecular Cloning, A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press (1989). More specifically, fractions of total RNA are run through the chromatographic resin, and mRNA is eluted therefrom with an elution buffer. The RNA which binds to the column is enriched for RNAs containing poly(A) tails and, therefore, eliminates contaminants, such as rRNA and partially degraded mRNAs. It is important that the purification be carried out successfully such that when cDNA is synthesized from the mRNA, higher yields of mRNA copies and less spurious copying of non-messenger RNAs occurs.

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Total RNA and polyadenylated RNA from the preparations were further fractionated on 1% formaldehyde gels, blotted to Nytran<sup>R</sup> membranes and analyzed to confirm that the enzymes in the cellulase complex were being induced as polyadenylated mRNA.

After isolating polyadenylated mRNA from total RNA, complementary DNA or cDNA is synthesized therefrom. The first strand of cDNA is synthesized using the enzyme RNA-dependent DNA polymerase (reverse transcriptase) to catalyze the reaction. Avian reverse transcriptase which is purified from the particles of an avian retrovirus or murine reverse transcriptase, which is isolated from a strain of E. coli that expresses a cloned copy of the reverse transcriptase gene of the Moloney murine leukemia virus can be used in the present invention. However, it is preferable to use the Moloney murine leukemia virus (M-MLV) reverse transcriptase to synthesize first strand cDNA from the polyadenylated mRNA population. The amount of cloned M-MLV reverse transcriptase required may vary depending on the amount of polyadenylated mRNA used in the synthesis reaction. Usually, about 200 U/ $\mu$ l of the reverse transcriptase is used per 2 to 10  $\mu$ g of mRNA per reaction.

Also present in the synthesis mixture is a primer to initiate synthesis of DNA. For cloning of cDNAs, any primer can be used, but it is preferable to use oligo(dT) containing 12-18 nucleotides in length, which binds to the poly(A) tract at the 3' terminus of eukaryotic cellular mRNA molecules. The primer is added to the reaction mixture in large molar excess so

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that each molecule of mRNA binds several molecules of oligo(dT)<sub>12-18</sub>. It is preferable to use about 12.5 µg of primer having a concentration of 0.5 mg/ml.

5 Besides the enzyme and primer, a buffer and dNTP mix containing dATP, dCTP, dGTP, and dTTP at a final concentration of 500 µM each usually completes the reaction cocktail. Any buffer can be used in the present invention for first strand cDNA synthesis that is compatible with this synthesis. It is preferable to  
10 use a buffering system consisting of 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl<sub>2</sub>, and 50 mM dithiothreitol. Generally, about 500 µl of buffer completes the synthesis solution.

15 After the first strand is synthesized, the second strand of cDNA may be synthesized by a variety of methods known in the art, such as hairpin-primed synthesis by denaturing the cDNA:mRNA complex, adding the Klenow fragment of E.coli DNA polymerase or reverse transcriptase, and then digesting the hairpin loop with  
20 nuclease S1 to obtain a double-stranded cDNA molecule, the Okayama and Berg method, the Gubler and Hoffman method, and the like. The Okayama and Berg method uses E. coli RNase H to randomly nick the mRNA, and the RNA is replaced in the nick translation reaction by  
25 catalysis with E. coli DNA polymerase I. In the Okayama and Berg method, mRNA is used to prime DNA synthesis by the E. coli DNA polymerase I.

The preferred method to synthesize the second strand of cDNA is a modified method of the Gubler and  
30 Hoffman procedure. This procedure uses E. coli RNase H, DNA Polymerase I, and DNA Ligase to form the second

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strand. Actually, two different methods of proceeding with the second strand synthesis can be used in the present invention. The first procedure uses RNase H to attack the RNA:DNA hybrid in a random fashion, producing nicks in addition to those produced by reverse transcriptase. If too many nicks are introduced into the RNA at the 5' end of the message before second strand synthesis commences, fragments may be produced that are too short to remain hybridized; thus, they will not be able to serve as primers. In addition, the 5'-most RNA oligomer which primes second strand DNA synthesis will continue to be degraded until only two ribonucleotides remain at the 5' end of the second strand DNA. These are substrates for the polymerase I RNase H activity, and the remaining nucleotides will be removed. This leaves the 3' end of the first strand cDNA single stranded, making it a substrate for the 3' exonuclease activity of Polymerase I. The result is a population of cDNAs, which are blunt-ended.

An alternative method relies on M-MLV reverse transcriptase to produce nicks 10 to 20 bases from the 5' end of the RNA in the hybrid. DNA polymerase I is then used for synthesis. Generally, about 500 units at a concentration of 10 U/ $\mu$ l of DNA polymerase I is used. After second strand synthesis, RNase H is added after removal of the DNA polymerase I to produce a duplex, which is entirely DNA, except for the surviving capped RNA 5' oligonucleotide.

The second-strand synthesis by either procedure set forth above usually takes place in the presence of a buffer and dNTP mix. Any buffering system that is

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known in the art for second strand cDNA synthesis can be used; however, it is preferable to use a buffering system containing 188 mM Tris-HCl, pH 8.3, 906 mM KCl, 100 mM  $(\text{NH}_4)_2\text{SO}_4$ , 46 mM  $\text{MgCl}_2$ , 37.5 mM dithiothreitol, and 1.5 mM NAD. The dNTP mix preferably contains 10 mM dATP, 10 mM dCTP, 10 mM dGTP, and 10 mM dTTP.

The second strand synthesis is carried out under known procedures set forth in the art. The preferred methods and reagents used to synthesize cDNA in the present invention are the BRL cDNA Synthesis System<sup>R</sup> (Bethesda Research Laboratories, Gaithersburg, Maryland) and the Librarian System (Invitrogen, San Diego, CA).

At this point a pool of cDNAs, a small portion of which code for the bgl1 gene, is present after second strand synthesis. Since amplification of only the specific bgl1 gene fragment in the cDNA pool is crucial for the isolation of the  $\beta$ -glucosidase gene, specific primers were designed to amplify the cDNA fragment encoding the T. reesei bgl1 gene in the polymerase chain reaction (PCR). The primers used are degenerate primers designed to hybridize to the cDNA of the bgl1 gene encoding the N-terminus and an internal CNBr fragment.

In general, it is difficult to isolate the bgl1 gene because the amino acid sequence of the protein does not contain sufficient amino acids which are coded for by unique nucleic acid triplets and thus any oligonucleotide used would be too degenerate to specifically amplify the bgl1 gene in the PCR reaction. However, in this invention, primers were designed by

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examining the amino acids of the region targeted for amplification of mature  $\beta$ -glucosidase and choosing regions, which will require a reduced degree of degeneracy in the genetic code. Codon bias in T. reesei for various other cellulase genes such as cbh1, cbh2, egl1, and the like was also taken into account when designing the oligonucleotide primers. More specifically, codon bias is based on various genes in the strain T. reesei which display a preferred nucleotide triplet encoding different amino acids. By analyzing this codon bias one can determine that a particular nucleotide sequence coding for an amino acid would be preferred. For example, the cbh1, cbh2 and egl genes from T. reesei prefer the CCU coding for the amino acid proline. Thus, when designing an oligonucleotide probe, the CUG sequence would be the preferred choice for leucine, rather than the other triplets (CUU, CUC, CUA, UUA and CUG) which code for leucine.

Furthermore, after selection of an N-terminal region and an internal region as primers for amplification purposes, the primers were designed by inserting a non-specific base inosine into the wobble position of the primer for the N-terminus and using a pool of sixteen variable primer sequences for the internal primer. Basically, the creation of degenerate primers is described by Compton in "Degenerate Primers For DNA Amplification" and Lee et al in "cDNA Cloning Using Degenerate Primers" in PCR Protocols: A Guide to Methods and Applications, published by Academic Press (1990).

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Using the primers described above, the cDNA sequences encoding the amino terminal region of the bgl1 gene is then selectively amplified using PCR. The amplification method consists of an initial, denaturing cycle of between about 5 to 15 minutes at 95°C, followed by a 1-7 minutes annealing step at a temperature between 35°C and 55°C and preferably between 45°C and 55°C and a 5-15 minutes polymerization cycle at 65°C. It is preferable, however, to use a 10 minute initial denaturing cycle, followed by 2 minutes of annealing at 50°C and a 10 minute, and preferably a 30 minute polymerization cycle at the aforescribed temperatures.

The amplified fragment is then identified via gel electrophoresis as a 700 bp cDNA segment. The amplified pool of cDNAs is then further fractionated on a polyacrylamide gel to obtain a more purified 700 bp cDNA fragment for cloning purposes. After elution of the 700 bp fragments from the gel, the 700 bp cDNA fragments are then cloned into phagemid vectors. Any cloning vector can be used to clone the cDNA bgl1 gene fragments, such as pUC18, pUC19, pUC118, pUC119, pBR322, pEMBL, pRSA101, pBluescript, and the like. However, it is preferable to use the cloning vectors pUC218 and pUC219, which are derived from pUC18 and pUC19 by insertion of the intergenic region of M13. The cloning vectors with the cDNA fragments containing the bgl1 gene are then used to transform E. coli strain JM101. After transformation, positive colonies containing the bgl1 gene were identified and DNA

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isolated therefrom using chloroform:phenol extraction and ethanol precipitation methods.

The nucleotide sequence of the subcloned cDNA 700 bp fragment is then determined by the dideoxy chain termination method described by Sanger et al using a Sequenase<sup>R</sup> reagent kit provided by U.S. Biochemicals.

From this nucleotide sequence it was determined that the subcloned 700 bp cDNA segment contained an open reading frame encoding 150 amino acids that overlapped a number of other sequenced peptides that were obtained following CNBr and proteolytic degradation of purified T. reesei  $\beta$ -glucosidase. Thus, it was confirmed that the cloned sequences encoded for the extracellular T. reesei  $\beta$ -glucosidase protein.

The cloning of the genomic version of the entire  $\beta$ -glucosidase gene was then undertaken by labelling the 700 bp bgl1 cDNA fragment with <sup>32</sup>P using the methods to label oligonucleotides described by Sambrook et al, supra. This probe is used to identify a 6.0 kb band on a Southern blot of Hind III digested genomic DNA from T. reesei.

The genomic DNA from T. reesei is prepared for Southern blot analysis by deproteinizing the genomic DNA, followed by treatment with ribonuclease A. The prepared genomic DNA is then cut with one of a variety of restriction enzymes such as Eco RI, Hind III and the like, run on a gel, Southern blotted and hybridized with the 700 bp cDNA labelled fragment of the bgl1 gene. From this analysis, it was determined that Hind III was the restriction enzyme of choice that can be used to clone the bgl1 gene.



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Hind III is then added to genomic DNA from the strain T. reesei and DNA is extracted therefrom. A sample from this digestion is run on an agarose gel and fractionated electrophoretically. The gel is then

5 Southern blotted and probed with the 700 bp cDNA probe. A 6.0 kb band was then identified on the Southern blot of Hind III digested genomic DNA. The remaining Hind III digested genomic DNA was then subjected to

10 preparative gel electrophoresis and DNA ranging in size from about 5.0 kb to 7.0 kb was eluted therefrom and cloned into a phagemid vector and used to transform E. coli JM101 to create a library. Any phagemid vector can be used such as those described above, however it is preferable to use pUC218. The colonies that

15 resulted from the transformation were then subjected to colony hybridization using the 700 bp cDNA fragment as a probe to identify those colonies that contained the cloned genomic DNA coding for bgl1. The positive colonies from the transformation are then picked and

20 the DNA isolated therefrom by methods known in the art.

The isolated DNA from such a positive colony is then digested with various restriction enzymes, both singly and in various combinations, and subjected to agarose gel electrophoresis. The resultant banding

25 pattern is then used to construct a restriction map of the cloned 6.0 kb genomic DNA from T. reesei. Enzymes used in the digestion include Eco RI, Sst I, Kpn I, Sma I, Bam HI, Xho I, Bgl II, Cla I, Xba I, Sal I, Pst I, Sph I, Hind III, Bal I, Pvu II and the like.

30 The same gel is then subject to Southern blot analysis using the same 700 bp bgl1 cDNA as a probe to

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identify which genomic restriction fragments shared  
homology with the bgl1 cDNA. Since the position of  
these homologous fragments can be determined relative  
to the restriction map of the 6.0 kb genomic fragment  
and also since the size of the  $\beta$ -glucosidase protein  
(74 kd) gives an estimated length of the gene as 2.1 kb  
(because average molecular weight of an amino acid is  
105 daltons, a 74 kd protein contains on average 705  
amino acids, which in turn is equal to 2,115 bp), then  
the mapping experiments confirmed that the entire bgl1  
gene is contained on the genomic Hind III clone.

Pvu II and Bal I restriction fragments ranging in  
size from 600 bp to 1500 bp hybridized with the 700 bp  
cDNA bgl1 clone and were thus chosen for subcloning  
into pUC218 phagemids. The nucleotide sequence was  
determined using the methods of Sanger et al, described  
above. The Pvu II and Bal I subclones were sequenced  
and the overlapping sequences of the subclones aligned  
until a single contiguous sequence totaling 3033 bp was  
obtained within which the nucleotide sequence of the  
bgl1 gene was determined on both strands and the  
position of two small introns was inferred by homology  
to introns of other genes of filamentous fungi. The  
amino acid sequence is also deduced as set forth in  
Figure 1.

The nucleotide sequence and deduced primary amino  
acid sequence of the entire T. reesei bgl1 gene is set  
forth in Figure 1. The predicted molecular weight of  
the encoded  $\beta$ -glucosidase protein is 74,341. A 31  
amino acid peptide precedes the mature amino terminus  
of  $\beta$ -glucosidase as deduced from the amino terminal

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peptide sequence. Within this peptide, there are three potential signal peptidase recognition sites consisting of Ala-X-Ala.

5 The primary amino acid sequence of B-glucosidase shows 7 potential N-linked glycosylation sites at positions 208, 310, 417, and 566, which shows the consensus Asn-X-Ser/Thr-X where X is not a proline. However, sites at positions 45, 566, and 658 have a proline residue in the consensus sequence and may or  
10 may not be glycosylated.

No unusual codon bias is observed in the bgl1 gene when compared to other cellulase genes. The bgl1 coding region is interrupted by two short introns of 70 bp and 64 bp, respectively. Both introns have splice  
15 site donor, splice acceptor, and lariat branch acceptor sites that show homology to the consensus splice signals emerging from T. reesei and other filamentous fungi.

Since the bgl1 gene from the T. reesei strain is  
20 identified and can be cloned, the next step is to produce a transformant that has extra copies of the bgl1 gene.

A selectable marker must first be chosen so as to enable detection of the transformed filamentous fungus.  
25 Different selectable markers may be used including argB from A. nidulans or T. reesei, amdS from A. nidulans, pyr4 from Neurospora crassa, A. nidulans or T. reesei, and pyrG from Aspergillus niger. The selectable marker can be derived from a gene, which specifies a novel  
30 phenotype, such as the ability to utilize a metabolite that is usually not metabolized by the filamentous

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fungi to be transformed or the ability to resist toxic shock effects of a chemical or an antibiotic. Also contemplated within the present invention are synthetic gene markers that can be synthesized by methods known in the art. Transformants can then be selected on the basis of the selectable marker introduced therein. Because T. reesei does not contain the amdS gene, it is preferable to use the amdS gene in T. reesei as a selectable marker that encodes the enzyme acetamidase, which allows transformant cells to grow on acetamide as a nitrogen source. In the case where the bglI gene is deleted from T. reesei, it is preferable to use the pyrG gene as a selectable marker.

The host strain used should be mutants of the filamentous fungi which lack or have a nonfunctional gene or genes corresponding to the selectable marker chosen. For example, if the selectable marker of argB is used, then a specific arg mutant strain is used as a recipient in the transformation procedure. Other examples of selectable markers that can be used in the present invention include the genes trp, pyr4, pyrG, trp1, oliC31, Bm1, pkiA, niaD, leu, and the like. The corresponding recipient strain must, therefore, be a mutant strain such as trp<sup>-</sup>, pyr<sup>-</sup>, leu<sup>-</sup>, and the like.

The mutant strain is derived from a starting host strain, which is any filamentous fungi strain. However, it is preferable to use a filamentous fungi over-producing mutant strain and particularly, a T. reesei overproducing strain described previously, since this strain secretes high amounts of proteins and, in particular, high amounts of cellulase enzymes. The

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selected mutant strain is then used in the transformation process. The preferred strain of T. reesei for use in deleting the bglI gene is RLP37 pyrG69, a uridine auxotroph.

5       The mutant strain of the selected filamentous fungi can be prepared by a number of techniques known in the art, such as the filtration enrichment technique described by Nevalainen in "Genetic improvement of  
10       enzyme production in industrially important fungal strains", Technical Research Center of Finland, Publications 26 (1985). Another technique to obtain the mutant strain is to identify the mutants under different growth medium conditions. For instance, the  
15       arg<sup>-</sup> mutants can be identified by using a series of minimal plates supplied by different intermediates in arginine biosynthesis. Another example is the production of pyr<sup>-</sup> mutant strains by subjecting the strains to fluoro-orotic acid (FOA). Strains with an intact pyr4 gene grow in an uridine medium and are  
20       sensitive to fluoro-orotic acid, and, therefore, it is possible to select pyr4<sup>-</sup> mutant strains by selecting for FOA resistance.

      The chosen selectable marker is then cloned into a suitable plasmid. Any plasmid can be used in the  
25       present invention for the cloning of the selectable marker such as pUC18, pBR322, and the like. However, it is preferable to use pUC100. The vector is created by digesting pUC100 with the restriction enzyme SmaI, and the 5' phosphate groups are then removed by  
30       digestion with calf alkaline phosphatase. The fragment vector is then purified by gel electrophoresis followed

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by electroelution from the isolated gel slice. The amdS gene from A. nidulans is isolated as a 2.4 kb SstI restriction fragment following separation of the vector sequences via known procedures such as those described  
5 by Hynes et al, Mol. Cell. Biol., 3, pp. 1430-1439 (1983). The 2.4 Kb SstI amdS fragment and the 2.7 Kb pUC100 vector fragment are then ligated together, and the ligation mix is then introduced into the E. coli host strain JM101.

10 Any plasmid can be used in the present invention for the insertion of the bgl1 gene, but it is preferable to use the pSAS plasmid.

pSASB-glu is constructed by digesting pSAS with the restriction enzyme Hind III and purifying the linear  
15 fragment via gel electrophoreses and electroelution. Into this Hind III treated pSAS vector fragment is ligated the 6.0 Kb Hind III fragment of T. reesei genomic DNA that contained all of the coding region of the bgl1 gene along with the sequences necessary for  
20 transcription and translation. Figure 2 illustrates the construction of pSASB-glu.

It is also possible to construct vectors that contain at least one additional copy of the bgl1 gene and to construct vectors in which the amino acid  
25 sequence of bgl1 gene has been altered by known techniques in the art such as site directed mutagenesis, PCR methods, and chemical mutation methods.

In another embodiment, the bgl1 gene of a  
30  $\beta$ -glucosidic filamentous fungi can be totally deleted and may be replaced with other known genes.

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Preferably, the replacing gene is homologous to the filamentous fungi so that the resulting recombinant microorganism does not express any heterologous protein. For example, potentially any T. reesei gene which clones for a selected marker and which has been cloned and thus identified in the genome, can replace the bglI gene in T. reesei using the techniques described herein.

On the other hand, the replacing gene does not necessarily have to be homologous. Specifically, for the deletion of the bglI gene in T. reesei, vectors containing heterologous gene which have been used are illustrated in Figures 3A and 3B. In Figure 3B, a pUC218 vector plasmid having the Aspergillus niger pyrG gene inserted therein is illustrated. A 6.0 Kb genomic Hin dIII fragment, known to contain the entire bglI gene, is cloned into the polylinker of pUC218. The coding region for the bglI gene is then removed from this plasmid using either unique BalI restriction sites (Fig. 3A) or unique ApaI and EcoRV restriction sites (Fig. 3B) situated at the very 5' and 3' end of the bglI open reading frame and replaced with an isolated 2412 bp Hin dIII/Bam HI restriction fragment containing the pyrG gene from Aspergillus niger. All restriction ends are made blunt by treatment with T4 DNA polymerase prior to ligation using T4 DNA ligase.

After a suitable vector is constructed, it is used to transform strains of filamentous fungi. Since the permeability of the cell wall in filamentous fungi (e.g., T. reesei) is very low, uptake of the desired DNA sequence, gene or gene fragment is at best minimal.

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To overcome this problem, the permeability of the cell wall can be increased or the DNA can be directly shot into the cells via a particle gun approach. In the particle gun approach, the DNA to be incorporated into the cells is coated onto micron size beads and these beads are literally shot into the cells leaving the DNA therein and leaving a hole in the cell membrane. The cell then self-repairs the cell membrane leaving the DNA incorporated therein. Besides this aforescribed method, there are a number of methods to increase the permeability of filamentous fungi cells walls in the mutant strain (i.e., lacking a functional gene corresponding to the used selectable marker) prior to the transformation process.

One method involves the addition of alkali or alkaline ions at high concentrations to filamentous fungi cells. Any alkali metal or alkaline earth metal ion can be used in the present invention; however, it is preferable to use either  $\text{CaCl}_2$  or lithium acetate and more preferable to use lithium acetate. The concentration of the alkali or alkaline ions may vary depending on the ion used, and usually between 0.05 M to 0.4 M concentrations are used. It is preferable to use about a 0.1 M concentration.

Another method that can be used to induce cell wall permeability to enhance DNA uptake in filamentous fungi is to resuspend the cells in a growth medium supplemented with sorbitol and carrier calf thymus DNA. Glass beads are then added to the supplemented medium, and the mixture is vortexed at high speed for about 30



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seconds. This treatment disrupts the cell walls, but may kill many of the cells.

Yet another method to prepare filamentous fungi for transformation involves the preparation of protoplasts. Fungal mycelium is a source of protoplasts, so that the mycelium can be isolated from the cells. The protoplast preparations are then protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, sodium chloride, magnesium sulfate, and the like. Usually, the concentration of these stabilizers varies between 0.8 M to 1.2 M. It is preferable to use about a 1.2 M solution of sorbitol in the suspension medium.

Uptake of the DNA into the host mutant filamentous fungi strain is dependent upon the concentration of calcium ion. Generally, between about 10 mM  $\text{CaCl}_2$  and 50 mM  $\text{CaCl}_2$  is used in an uptake solution. Besides the need for calcium ions in the uptake solution, other items generally included are a buffering system such as TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA) or 10 mM MOPS, pH 6.0 buffer (morpholinepropane-sulfonic acid), and polyethylene glycol (PEG). The polyethylene glycol acts to fuse the cell membranes, thus permitting the contents of the mycelium to be delivered into the cytoplasm of the filamentous fungi mutant strain and the plasmid DNA is transferred to the nucleus. This fusion frequently leaves multiple copies of the plasmid DNA tandemly integrated into the host chromosome. Generally, a high concentration of PEG is used in the uptake solution. Up to 10 volumes of 25% PEG 4000 can be used in the uptake solution. However, it is

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preferable to add about 4 volumes in the uptake solution. Additives such as dimethyl sulfoxide, heparin spermidine, potassium chloride, and the like may also be added to the uptake solution and aid in transformation.

5 Usually a suspension containing the filamentous fungi mutant cells that have been subjected to a permeability treatment or protoplasts at a density of  $10^8$  to  $10^9$ /ml, preferably  $2 \times 10^8$ /ml, are used in transformation. These protoplasts or cells are added to the uptake solution, along with the desired transformant vector containing a selectable marker and other genes of interest to form a transformation mixture.

15 The mixture is then incubated at  $4^\circ\text{C}$  for a period between 10 to 30 minutes. Additional PEG is then added to the uptake solution to further enhance the uptake of the desired gene or DNA sequence. The PEG may be added in volumes of up to 10 times the volume of the transformation mixture, preferably, about 9 times.

20 After the PEG is added, the transformation mixture is then incubated at room temperature before the addition of a sorbitol and  $\text{CaCl}_2$  solution. The protoplast suspension is then further added to molten aliquots of a growth medium. This growth medium contains no uridine and selectively permits the growth of transformants only. The subsequent colonies were transferred and purified on a growth medium depleted of sorbitol.

25 At this stage, stable transformants can be distinguished from unstable transformants by their

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5 faster growth rate and the formation of circular colonies with a smooth rather than ragged outline on solid culture medium. Additionally, in some cases, a further test of stability can be made by growing the transformants on solid non-selective medium, harvesting the spores from this culture medium and determining the percentage of these spores which will subsequently germinate and grow on selective medium.

10 In order to ensure that the transformation took place by the above-described methods, further analysis is performed on the transformants such as Southern blotting and autoradiography. Using the same basic procedures set forth above, the entire bgl1 gene can be deleted from a vector and transformed into filamentous  
15 fungi strains or the bgl1 gene can be altered and transformed into filamentous fungi strains.

After confirmation that the transformed strains contained at least one additional copy of the bgl1 gene, an altered bgl1 gene or the transformants  
20 contained a deleted bgl1 gene, the strains are further cultured under conditions permitting these transformants to propagate. The transformants can then be isolated from the culture media and used in a variety of applications which are described below.  
25 Alternatively, the transformants can be further fermented and a recombinant fungal cellulase composition can be isolated from the culture media. Since, for example, the transformants produced by the present invention can express enhanced, deleted or  
30 altered extracellular  $\beta$ -glucosidase in the fermentation medium, fungal cellulase compositions can be isolated

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from the medium. Usually, the isolation procedure involves centrifuging the culture or fermentation medium containing the transformants and filtering by ultrafiltration the supernatant to obtain a recombinantly produced fungal cellulase composition. Optionally, an antimicrobial agent can be further added to the composition prior to use in the variety of applications described below. Examples of microbial agents that can be added are sodium azide, sodium benzoate and the like.

Confirmation that the transformants produced by the process of the present invention had enhanced activity on cellobiose, the following experiment was performed. In this experiment 50 mg of cellobiose which was suspended in 1.0 ml of phosphate buffer (pH 5.0) and was reacted with the fermentation product produced by the transformant (65.5 mg/ml protein) using a fermentation product from a normal nonmutant T. reesei strain as a control (135.0 mg/ml protein). The results of cellobiase activity under conditions of initial rate, are set forth in Table I below:

TABLE I

Product	Protein (mg/ml)	Activity on Cellobiose <u><math>\mu</math>mole glucose</u> mg protein
Control	135.0	6
Product produced by the present invention	65.5	33

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The results from this experiment indicate that the fermentation product produced by the transformants of the present invention has over five times the specific activity on the substrate, cellobiose, compared to a nonmutant T. reesei control strain.

Moreover, Figures 7 and 8 confirm that hydrolysis is enhanced for the substrates Avicel and PSC (note: PSC is a phosphoric acid swollen cellulose obtained by treating Avicel with phosphoric acid) using 1.0% enzyme/substrate. In the experiment, PSC or Avicel was suspended in 2 mls of 50 mM sodium acetate buffer, pH 4.8, and incubated at 40° under non-agitated conditions for up to 24 hours. Soluble reducing sugar was measured by the method of Nelson and Somogyi. From these figures it is further demonstrated that the enhanced recombinant  $\beta$ -glucosidase fermentation product produced from transformants according to the present invention, has an increased rate and extent of hydrolytic activity on the various substrates compared to the standard Cyt-123 control (on average 20% higher activity). The Cyt-123 control is the product obtained from a T. reesei cellulase over-production strain subjected to fermentation on an industrial scale.

The enriched transformants can be used in a variety of different applications. For instance, some  $\beta$ -glucosidases can be further isolated from the culture medium containing the enhanced transformants and added to grapes during wine making to enhance the potential aroma of the finished wine product. Yet another application can be to use  $\beta$ -glucosidase in fruit to

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enhance the aroma thereof. Alternatively, the isolated recombinant fermentation product containing enhanced  $\beta$ -glucosidase can be used directly in food additives or wine processing to enhance the flavor and aroma.

5        Since the rate of hydrolysis of cellulosic products is increased by using the transformants having at least one additional copy of the bgl1 gene inserted into the genome, products that contain cellulose or heteroglycans can be degraded at a faster rate and to a  
10       greater extent. Products made from cellulose such as paper, cotton, cellulosic diapers and the like can be degraded more efficiently in a landfill. Figure 9 illustrates the use of an increased  $\beta$ -glucosidase preparation isolated from the fermentation medium  
15       containing transformants having at least one additional copy of the bgl1 gene inserted into the genome compared to a non-enhanced Cyt 123 standard (defined above) on a cellulosic diaper product. This hydrolysis experiment was performed using 0.4 mg of the standard and the  
20       fermentation product per 100 mg of substrate (the cellulosic diaper). The experiment was run at 50°C over a period of five hours and the glucose concentration was measured, in duplicate, at various time intervals. This curve illustrates an increased  
25       rate of hydrolysis for the product produced by the fermentation product derived from the transformant having additional copies of bgl1, compared to the standard. It was also determined that the diaper derived fibers were about 14% insoluble in aqueous  
30       solution. Thus, the fermentation product obtained from the transformants or the transformants alone can be

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used in compositions to help degrade by liquefaction a variety of cellulose products that add to overcrowded landfills.

Simultaneous saccharification and fermentation is a process whereby cellulose present in biomass is converted to glucose and, at the same time and in the same reactor, yeast strains convert the glucose into ethanol. Yeast strains that are known for use in this type of process include B. clausenii, S. cerevisiae, Cellulolyticus acidothermo-philium, C. brassicae, C. lustinaniae, S. uvarum, Schizosaccharomyces pombe and the like. Ethanol from this process can be further used as an octane enhancer or directly as a fuel in lieu of gasoline which is advantageous because ethanol as a fuel source is more environmentally friendly than petroleum derived products. It is known that the use of ethanol will improve air quality and possibly reduce local ozone levels and smog. Moreover, utilization of ethanol in lieu of gasoline can be of strategic importance in buffering the impact of sudden shifts in non-renewable energy and petro-chemical supplies.

Ethanol can be produced via saccharification and fermentation processes from cellulosic biomass such as trees, herbaceous plants, municipal solid waste and agricultural and forestry residues. However, one major problem encountered in this process is the lack of  $\beta$ -glucosidase in the system to convert cellobiose to glucose. It is known that cellobiose acts as an inhibitor of cellobiohydrolases and endoglucanases and thereby reduces the rate of hydrolysis for the entire cellulase system. Therefore, the use of increased

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$\beta$ -glucosidase activity to quickly convert cellobiose into glucose would greatly enhance the production of ethanol. To illustrate this point, cytolase 123 and the fermentation product produced by the transformants (normalized to cytolase on a total protein basis) according to the present invention under fermentation conditions were compared for their ability to hydrolyze crude paper fractions composed of 50-60% cellulose from a fiber fraction (RDF) of municipal solid waste (MSW). Such suspensions were in 50 mM sodium acetate buffer, pH 4.8 to 5.0, and equilibrated at 30°C. The flasks were then dosed with 4% Saccharomyces cerevisiae and sampled periodically to 80 hours. The ethanol production yield was then measured. The following Table II illustrates that increased ethanol production is possible using the increased  $\beta$ -glucosidase preparation from the present invention using municipal solid waste preparations as the cellulosic source.

TABLE II

Dosage	Grams/Liter Ethanol	
	Cytolase 123	High $\beta$ -Glu Prep
10	2.1	5.0
20	5.3	7.2
30	6.9	8.8
40	8.0	9.3
50	8.5	9.3
60	8.5	9.3



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From Table II it can be clearly seen that the enhance  $\beta$ -glucosidase preparation prepared according to the present invention enhances the production of ethanol compared to a cytolase 123 control, especially at the lower protein concentrations.

In yet another embodiment of this invention, the deletion of the bgl1 gene from T. reesei strains would be particularly useful in preparing cellulase compositions for use in detergents and in isolating cellooligosaccharides (e.g., cellobiose).

The cellulase enzymes have been used in a variety of detergent compositions to enzymatically soften clothes and to provide color restoration. However, it is known in this art that use of cellulase enzymes can impart degradation of the cellulose fibers in clothes. One possibility to decrease the degradation effect is to produce a detergent that does not contain  $\beta$ -glucosidase. Thus, the deletion of this protein would effect the cellulase system to inhibit the other components via accumulation of cellobiose. The modified microorganisms of this invention are particularly suitable for preparing such compositions because the bgl1 gene can be deleted leaving the remaining CBH and EG components thereby resulting in color restoration and improved softening benefits in the composition without degradative effects.

The detergent compositions of this invention may employ, besides the cellulase composition (deleted in  $\beta$ -glucosidase), a surfactant, including anionic, non-ionic and ampholytic surfactants, a hydrolase, building agents, bleaching agents, bluing agents and fluorescent

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dyes, caking inhibitors, solubilizers, cationic surfactants and the like. All of these components are known in the detergent art. For a more thorough discussion, see U.S. Application Serial No. 07/593,919  
5 entitled "Trichoderma reesei Containing Deleted Cellulase Genes and Detergent Compositions Containing Cellulases Derived Therefrom", and U.S. Serial No. 07/770,049, filed October 4, 1991 and entitled  
10 "Trichoderma reesei Containing Deleted and/or enriched Cellulase and other enzyme Genes and Cellulase Compositions Derived Therefrom" both of which are incorporated herein by reference in their entirety.

In yet another embodiment, the detergent compositions can also contain enhanced levels of  
15  $\beta$ -glucosidase or altered  $\beta$ -glucosidase. In this regard, it really depends upon the type of product one desires to use in detergent compositions to give the appropriate effects.

Preferably the cellulase compositions are employed  
20 from about 0.00005 weight percent to about 5 weight percent relative to the total detergent composition. More preferably, the cellulase compositions are employed from about 0.01 weight percent to about 5 weight percent relative to the total detergent  
25 composition and even more preferably, from about 0.05 to about 2 weight percent relative to the total detergent composition.

Deletion of the bgl1 gene would also provide accumulation of celooligosaccharides (e.g.,  
30 cellobiose) in cellulosic solutions treated with cellulase system, which can be purified therefrom. In

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this regard, the present invention presents the possibility to isolate cellooligosaccharides employing microorganisms in an easy and effective manner.

5 Cellooligosaccharides are useful in assaying cellulase enzymes for enzymatic activity and are also useful in the synthesis of ethanol and glucose. Moreover, it is contemplated that such oligosaccharides would also be useful as food additives, chemical intermediates, etc.

10 Heretofore, the use of cellulase containing  $\beta$ -glucosidase to prepare cellooligosaccharides required the deactivation of  $\beta$ -glucosidase by adjusting the pH of the solution to less than about 4 and generally to around 3.8. At this pH, the  $\beta$ -glucosidase is generally  
15 inactivated. However, at this pH, the other enzyme components of cellulase are generally less active as compared to their optimum pHs and, accordingly, such a reduction of pH to inactivate the  $\beta$ -glucosidase necessarily results in a less efficient process.

20 On the other hand, the use of cellulase compositions free of  $\beta$ -glucosidase as per this invention provides a facile means for preparing cellooligosaccharides at a pH of from about 4.5 to about 8; preferably at a pH of from about 4.5 to about  
25 6 and most preferably at the pH optimum for the cellulase composition employed. In this embodiment, the invention is directed to a process for producing cellooligosaccharides which comprises contacting cellulose containing materials (i.e., materials  
30 containing at least 20% cellulose and preferably at least 50% cellulose) with a cellulase composition free

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of  $\beta$ -glucosidase at a pH of from about 4.5 to about 8. Additionally, cellulase compositions containing reduced amounts of  $\beta$ -glucosidase can be obtained by mixing the cellulase produced by a  $\beta$ -glucosidic filamentous fungi and the cellulase produced by a  $\beta$ -glucosidic filamentous fungi which has been modified to be incapable of producing  $\beta$ -glucosidase.

Moreover, the present invention also contemplates the use of the  $\beta$ -glucosidase nucleotide sequence of T. reesei to design various probes for the identification of the extracellular  $\beta$ -glucosidase gene in other filamentous fungi. In this regard, the entire nucleotide sequence of the bgl1 gene can be used or a portion thereof to identify and clone out the equivalent genes from other filamentous fungi. The sources of filamentous fungi include those fungi from the genus Trichoderma, Aspergillus, Neurospora, Humicola, Penicillium and the like. More particularly, the preferred species include Trichoderma reesei, Trichoderma viridae, Aspergillus niger, Aspergillus oryzae, Neurospora crassa, Humicola grisea, Humicola insolens, Penicillium pinophilum, Penicillium oxalicum, Aspergillus phoenicis, Trichoderma koningii and the like. Due to the species homology of the bgl1 gene, filamentous fungi equivalent genes are easily identified and cloned. Indicative of this are Figures 10A and 10B which illustrate autoradiograph of A. nidulans and N. crassa (Figure 10A) and H. grisea (Figure 10B) DNA digested with Hind III and Eco RI and further were blotted and probed with a  $P^{32}$  labeled Hind III 6.0 kb bgl1 DNA fragment containing the bgl1 gene

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of T. reesei. These autoradiographs clearly illustrate that a DNA fragment containing the bgl1 gene of T. reesei can be used to identify the extracellular bgl1 gene in other fungi.

5        Thus the bgl1 gene of other filamentous fungi may be cloned by the methods outlined above using the  $p^{32}$  labelled T. reesei bgl1 gene as a probe. Once the genes of other filamentous fungi are cloned, they can be used to transform the filamentous fungi from which  
10       the gene was derived or other filamentous fungi to overproduce  $\beta$ -glucosidase by the methods described above. Alternatively, the cloned bgl1 genes from the other filamentous fungi can be used by the methods described above to delete or disrupt the bgl1 gene in  
15       the genome of the filamentous fungi from which the bgl1 gene was originally cloned.

      In order to further illustrate the present invention and advantages thereof, the following specific examples are given, it being understood that  
20       the same are intended only as illustrative and in nowise limitative.

#### Example 1

##### Isolation of Total RNA from Trichoderma reesei

      A Trichoderma reesei culture which over produces  
25       cellulases was specifically induced for cellulase using sophorose, a  $\beta$ ,1-2 diglucoside as described by Gritzali, 1977. The starting strain of Trichoderma reesei is a cellulase over-production strain (RL-P37) developed by mutagenesis by the methods described by  
30       Sheir-Neiss, G. and Montenecourt, B.S., Appl.

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Microbiol. Biotechnol., Vol. 20 (1984) pp. 46-53. A mycelial inoculum of T. reesei, from growth on potato dextrose agar (Difco), was added into 50 ml of Trichoderma basal medium containing 1.40 grams/liter

5 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0 grams/liter KH<sub>2</sub>PO<sub>4</sub>, 0.30 grams/liter MgSO<sub>4</sub>, 0.30 grams/liter urea, 7.50 grams/liter BactoPeptone, 5.0 ml/liter, 10% Tween - 80, 1.0 ml/liter trace elements-EFG, pH 5.4, which was filtered through a 0.2 micron filter in a 250 ml baffled flask.

10 This culture was incubated at 30°C for 48 hours with vigorous aeration. Five milliliter aliquots were taken from the culture and added to 25 ml of fresh basal medium in seven 250 ml flasks. These were subsequently grown for 24 hours at 30°C. All cultures were

15 centrifuged in a benchtop clinical centrifuge at 2400 x g for 10 minutes. The mycelial pellets were washed three times in 50 mls of 17 mM KHPO<sub>4</sub> buffer (pH 6.0). Lastly, the mycelia were suspended in six flasks containing 50 ml of 17 mM KHPO<sub>4</sub> buffer with the addition

20 of 1 mM sophorose and a control flask containing no sophorose. The flasks were incubated for 18 hours at 30°C prior to harvesting by filtration through Miracloth (Calbiochem). The excess medium was then squeezed out and the mycelial mat was placed directly

25 into liquid nitrogen and may be stored at -70°C for up to one month. The frozen hyphae were then ground in an electric coffee grinder that was prechilled with a few chips of dry ice until a fine powder was obtained. The powder was then added to about 20 ml of an extraction

30 buffer containing 9.6 grams of p-aminosalicylic acid dissolved in 80 ml of DEP-treated water, 1.6 grams of

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triisopropyl naphthalene sulfonic acid dissolved in 80 ml of DEP-treated water, 24.2 grams Tris-HCl, 14.6 grams NaCl, 19.09 grams EDTA, which was diluted to 200 ml total volume with DEP-treated water and the pH was  
5 adjusted to 8.5 with NaOH. After addition of the extraction buffer, 0.5 volumes of TE-saturated phenol was also added thereto, and the extraction mixture was placed on ice. One quarter volume of chloroform was then added to the extraction mixture, and the mixture  
10 was shaken for two minutes. The phases were then separated by centrifugation at 2500 rpm. The aqueous phase was removed and placed in a centrifuge tube, which contained a few drops of phenol in the bottom of said tube. The tube was placed on ice. The organic  
15 phase was then reextracted with 2.0 ml of extraction buffer and placed in a 68°C water bath for 5 minutes to release the RNA trapped in polysomes and at the interface of the extraction mixture. The extracted mixture was then centrifuged, and the aqueous phase  
20 removed and pooled with the other aqueous fraction.

The entire aqueous fractions were then extracted with phenol-chloroform (1:1 v/v) for 4 to 5 times until there was no longer any protein seen visually at the interface. Then 0.1 volume of 3 M sodium acetate, pH  
25 5.2 (made with DEP water and autoclaved) and 2.5 volumes of 95% was added to the organic extracts, and the extracts were frozen at -20°C for 2 to 3 hours. Alternatively, the RNA was precipitated using 2 M lithium acetate. The RNA was then pelleted by  
30 centrifugation at 12,000 rpm for 20 minutes. The pelleted RNA was then resuspended in DEP-water with an

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RNase inhibitor to a final concentration of 1 unit per  $\mu$ l. To determine whether the genes encoding the enzymes were being induced, total RNA was analyzed.

#### Analysis of Total RNA Preparation

5           To confirm whether the genes encoding the enzymes of the cellulase complex were being induced, total RNA was analyzed by Northern blotting as described by Sambrook et al, supra using a  $P^{32}$  fragment of the T. reesei cbh2 gene as a probe. The cbh2 clone was  
10 isolated using the methods described by Chen et al in "Nucleotide Sequence and Deduced Primary Structure of Cellobio-hydrolase II from Trichoderma reesei", Biotechnology, Vol. 5 (March 1987), incorporated herein by reference. Site directed mutagenesis (Sambrook et  
15 al., supra) was performed on the cbh2 clone and a Bgl II site was placed at the exact 5' end of the opening reading frame and an Nhe I site at the exact 3' end. The Bgl II/Nhe I coding sequence was then cloned into a pUC218 phagemid. For use as a probe, the cbh2 fragment  
20 was digested with Bgl II/Nhe I and isolated by gel electrophoresis. The results indicated that the level of cbh2 specific mRNA reached a peak at 14-18 hours post induction. The total RNA from 14, 18 and 22 hours was then pooled.

25

#### Example 2

##### Purification of Polyadenylated mRNA

mRNA was then isolated from the pooled fraction of total RNA set forth above using oligo (dT) cellulose chromatography. Oligo(dT) cellulose (type 3 from



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Collaborative Research, Lexington, MA) is first equilibrated with Oligo(dT) binding buffer containing 0.01 M Tris-HCl, pH 7.5, 0.5 M NaCl, and 1 mM EDTA, then aliquots of 25-300 mg were added to 1.5 ml microfuge tubes. RNA dissolved in 1 ml of binding buffer was added and allowed to bind for 15 min. with gentle shaking. The suspensions were centrifuged at 1500 g for 3-5 min., washed 3-5 times with 1 ml of binding buffer, and then washed 3 times with 400  $\mu$ l of elution buffer containing 0.01 M Tris-HCl, pH 7.5, and 1 mM EDTA. The eluates were pooled, readjusted to 0.5 M NaCl, rebound, and reeluted with three washes of elution buffer. The final three elution buffer washes were pooled and mRNA was recovered by ethanol precipitation.

Analysis of Total RNA and polyadenylated mRNA

Total RNA and the polyadenylated RNA were fractionated on 1% formaldehyde-agarose gels using 10  $\mu$ g of RNA for each lane, blotted to Nytran<sup>R</sup> membranes and analyzed by the Northern blot method described by Thomas in "Hybridization of denatured RNA and Small DNA fragments transferred to Nitrocellulose", Proc. Natl. Acad. Sci. USA, Vol. 77 (1980), pp. 5201-5205.

Briefly, this procedure involves denaturing RNA (up to 10  $\mu$ g/8  $\mu$ l reaction) by incubation in 1 M glyoxal/50% (vol/vol) Me<sub>2</sub>SO/10 mM sodium phosphate buffer, pH 7.0 at 50°C for 1 hr. The reaction mixture was cooled on ice and 2  $\mu$ l of sample buffer containing 50% (vol/vol) glycerol, 10 mM sodium phosphate buffer at 7.0 and bromophenol blue was added. The samples

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were electro-phoresed on horizontal 1% formaldehyde-agarose gels in 10 mM phosphate buffer, pH 7.0 at 90 v for 6 hours.

5 The glyoxylated RNA was transferred from agarose gels to nitrocellulose by using 3 M NaCl/0.3 M trisodium citrate (20X NaCl/cit). After electro-phoresis, the gel was placed over two sheets of Whatman 3 MM paper which was saturated with 20X NaCl/cit. Nitran<sup>R</sup> membrane was wetted with water, equilibrated  
10 with 20X NaCl/cit and laid over the gel. The gel was then covered with two sheets of Whatman 3 MM paper and a 5 to 7 cm layer of paper towels, a glass plate and a weight. Transfer of the RNA was completed in 12-15 hours. The blots were then dried under a lamp and  
15 baked in a vacuum for over 2 hrs. at 80°C.

The membranes were probed with a cbh2 probe to verify that the polyadenylated mRNA pool contained cbh2 mRNA and by inference the genes encoding the enzymes of the cellulase complex were indeed induced.

20

### Example 3

#### Synthesis of cDNA

##### A. First Strand Synthesis

Synthesis of cDNA was performed using the BRL cDNA Synthesis System<sup>R</sup> (Bethesda Research Laboratories, Md.)  
25 according to the instructions of the manufacturer. To a sterile, DEPC-treated tube in ice was added 10 µl of 5X First Strand Buffer containing 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>, 50 mM DTT, 2.5 µl 10 mM dNTP Mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM  
30 dTTP), 5 µl Oligo (dT)<sub>12-18</sub> (0.5) mg/ml, 10 µl of mRNA

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at 0.5 mg/ml and 20  $\mu$ l diethylpirocarbmate(DEPC)-  
treated water to create a final composition containing  
50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM  $MgCl_2$ , 10 mM  
dithiothreitol, 500  $\mu$ M each dATP, dCTP, dGTP and dTTP,  
5 50  $\mu$ g/ml Oligo (dT)<sub>12-18</sub>, 100  $\mu$ g/ml polyadenylated RNA  
and 10,000 U/ml cloned M-MLV reverse transcriptase. A  
control run was also run simultaneously using 10  $\mu$ l of  
a 2.3 kb control RNA (0.5 mg/ml) in lieu of the mRNA.

The reaction was initiated by adding 2.5  $\mu$ l of  
10 Molony murine leukemia virus (M-MLV) reverse  
transcriptase (100 Units/ $\mu$ l) to the mRNA tube and the  
control RNA. The samples were mixed. All reaction  
tubes were incubated at 37°C for one hour and then  
placed on ice.

15 A small aliquot from the reaction mixture was run  
on a gel to confirm its presence and quantity. The  
yield obtained was about 2-6  $\mu$ g.

#### B. Second Strand Synthesis

To the control tube on ice after first strand  
20 synthesis was added 230.6  $\mu$ l DEPC-treated water, 6  $\mu$ l  
10 mM dNTP mix, 32  $\mu$ l 10X second strand buffer  
containing 188 mM Tris-HCl, pH 8.3, 906 mM KCl, 100 mM  
( $NH_4$ )<sub>2</sub> SO<sub>4</sub>, 46 mM  $MgCl_2$ , 37.5 mM dithiothreitol, 1.5 mM  
NAD, 8  $\mu$ l E. coli DNA Polymerase I (10  $\mu$ /ml), 1.4  $\mu$ l  
25 E. coli RNase H and 1  $\mu$ l E. coli DNA ligase (100  
units).

To the first strand synthesis of the sample was  
added on ice 289.5  $\mu$ l of DEPC-treated water, 7.5  $\mu$ l 10  
mM dNTP mix, 40  $\mu$ l 10X second strand buffer, 10  $\mu$ l

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E. coli DNA Polymerase I, 1.75  $\mu$ l E. coli RNaseH and 1.25 E. coli DNA ligase, to create a final composition containing 25 mM Tris-HCl (pH 8.3), 100 mM KCl, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 5 mM  $\text{MgCl}_2$ , 250  $\mu$ M each dATP, dCTP, dGTP, dTTP, 0.15 mM NAD, 5 mM Dithiothreitol, 250 U/ml DNA Polymerase I, 8.5 U/ml RNase H, and 30 U/ml DNA Ligase. Both the control tube and the sample tube were vortexed gently and incubated for 2 hours at 16°C. After incubation, both tubes were placed on ice.

The sample tube was then extracted with 415  $\mu$ l of phenol and ethanol precipitated. The pellet was dissolved in 200  $\mu$ l of sterile TE buffer (10 mM Tris-HCl pH 7.5, 1 mM  $\text{Na}_2\text{EDTA}$ ) and reprecipitated from 7.5 M ammonium acetate with ethanol.

An aliquot of the sample was further analyzed by gel electrophoresis to check for purity. The yield of the synthesis was about 4.0  $\mu$ g.

The remaining control sample was further extracted with phenol and ethanol precipitated as described above for the sample. After dissolving the pellet in 200  $\mu$ l of sterile TE buffer, reprecipitating the sample from ammonium acetate with ethanol, and redissolving the dry pellet in 20  $\mu$ l of sterile TE buffer, 2  $\mu$ l of the solution was then further analyzed by gel electrophoresis to check for purity.

#### Example 4

##### Amplification of bgl1 cDNA Sequences

Amplification of the cDNA fragments encoding a portion of the T. reesei  $\beta$ -glucosidase gene, bgl1, was performed using the polymerase chain reaction (PCR)

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method with Taq<sup>R</sup> polymerase and a Perkin Elmer Cetus Thermal Cyclor<sup>R</sup>.

The reaction mixture was formed by mixing 76  $\mu$ l deionized water, 10  $\mu$ l of a 10X mixture of buffer  
5 containing 166 mM (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, 670 mM Tris-HCl, pH 8.8, 67 mM MgCl<sub>2</sub>, 67  $\mu$ M EDTA, 10 mM  $\beta$ -mercaptoethanol, 10  $\mu$ l dimethylsulfoxide and 1.7 mg/ml BSA diluted to a total volume of 1.0 ml with deionized water, 8  $\mu$ l of 2 dNTPs (each), 1  $\mu$ l 5' oligonucleotide primer, 1  $\mu$ l 3'  
10 internal oligonucleotide primer, 1.0  $\mu$ g cDNA diluted in 3  $\mu$ l deionized water, and 1  $\mu$ g Taq<sup>R</sup> polymerase.

The amplification method consists of an initial denaturing cycle at 95°C for 10 minutes, followed by a two minute annealing step at 50°C and a 10 minute  
15 polymerization cycle at 65°C, for an additional 30 cycles.

#### A. Oligonucleotide Primers

The oligonucleotide primers used to amplify the cDNA fragment encoding the T. reesei bgl1 gene were  
20 designed based on the degeneracy of the genetic code for the selected amino acids for an N terminal region of the bgl1 gene and an internal oligonucleotide. The 5' oligonucleotide primer consisted of the sequence:

5' GCI GTI CCT CCT GCI GG 3'

25 wherein I = inosine.

The internal 3' oligonucleotide primer consisted of a pool of 16 x 21 oligonucleotides. This pool was based on various derivations of the following sequences:

30 5' GTT G/ATT ICC G/ATT G/AAA G/ATC TGT 3'

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Example 5Subcloning of PCR Generated Fragments

Ninety  $\mu$ l of each reaction mix was fractionated on 4% polyacrylamide gels in 1X TBE, the major band was excised and eluted from the gel slice as described by Sambrook et al, supra. The eluted DNA fragment was precipitated in ethanol and resuspended in 15  $\mu$ l of TE buffer (10 mM Tris, 1 mM EDTA). Each 1-2  $\mu$ g DNA fragment was then treated with 0.5 mM ATP and  $T_4$  polynucleotide kinase to phosphorylate the 5' end of each fragment following by the procedures of Sambrook et al, supra. Blunt ends were generated by adding 3  $\mu$ l of 10X  $T_4$  polymerase buffer (330 mM Tris-acetate at pH 7.9, 660 mM potassium acetate, 100 mM magnesium acetate, 1  $\mu$ l of 2.5 mM dNTPS, 1  $\mu$ l of  $T_4$  DNA polymerase and 5  $\mu$ l of distilled water). The blunt-end reaction mixture was then incubated at 37° for 60 minutes. The reaction was stopped by addition of EDTA to a final concentration of 1 mM EDTA and the sample was further heated for 10 minutes at 65°C.

The blunt-end DNA fragments were then ligated with SmaI cleaved and dephosphorylated pUC218 which had been infected with M13X07 as described by Sambrook et al, supra. The cloning vectors pUC218 and pUC219 were derived from pUC118 and pUC119 by insertion of the Bgl II, Cla I and Xho I polylinker as described by Korman et al in "Cloning, Characterization, and expression of two  $\alpha$ -amylase genes from Aspergillus niger var. awamori", Current Genetics, Vol. 17, pp. 203-212, (1990).

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The aforescribed phagemid was then used to transform E. coli strain JM101 as described by Yarnisch et al in "Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 Vectors", Gene, Vol. 1197, pp. 103-119 (1985).

#### Example 6

##### Isolation of cDNA Subcloned Fragment

The transformed strain was inoculated in 1.5 ml of 2YT broth in a tube which had been previously inoculated with 15  $\mu$ l of saturated E. coli JM101. The culture was grown for 8 hours under shaking at 37°C.

The culture mixture was then spun at 6000 rpm for 5 minutes, and the supernatant was poured off into another tube. To the supernatant 300  $\mu$ l of 2.5 M NaCl, 20% PEG was added, and the solution was mixed. The mixture was then incubated at room temperature for 15 minutes.

The solution was then spun for 5 minutes in a microfuge, and the supernatant was aspirated off. The solution was vortexed once again, and the supernatant was further aspirated off.

100  $\mu$ l of equilibrated phenol was added to the tube, and the tube was vortexed. 100  $\mu$ l of chloroform was added, and once again the tube was vortexed. The tube was heated at 55°C for 5 minutes, mixed, and microfuged an additional 5 minutes.

160  $\mu$ l of the supernatant was then pipetted off and transferred to a clear tube. 20  $\mu$ l of 1N NaOAC, pH 4.5, and 400  $\mu$ l of 95% ETOH were added to the supernatant, and the solution was mixed and frozen on

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dry ice for 5 minutes. The tube was then spun for an additional 15 minutes, and the supernatant was aspirated off.

5        1000  $\mu$ l of 70% ethanol was added to the tube, and the tube was spun for an additional 2 minutes and reaspirated. The mixture was spun once more under vacuum for 4 minutes, and the pellet was resuspended in 15  $\mu$ l TE buffer.

#### Example 7

10        Determination of the Nucleotide Sequence  
          of 700 bp cDNA fragment

15        The nucleotide sequence of the 700 bp cDNA fragment was determined using the dideoxy DNA sequencing method described by Sanger et al, "DNA Sequencing with chain terminating inhibitors", Proc. Natl. Acad. Sci. U.S.A., Vol. 74 (1977), p. 5463, using the Sequenase<sup>R</sup> reagent kit (U.S. Biochemicals).

#### Example 8

##### Analysis of bgl1 gene

20        A. Sequence Analysis

Nucleotide sequencing was done by the dideoxy chain termination method of Sanger et al (1977) using the Sequenase<sup>R</sup> reagent kit (U.S. Biochemicals).

B. Amino Acid Sequencing

25        A 2.5-nmol sample of the reduced and carboxy-methylated  $\beta$ -glucosidase preparation purified (per Chirico and Brown, European Journal of Biochem., Vol.



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165, pp. 333 et seq.) was subjected to N-terminal sequencing on a proprietary multiphase sequencer.

To a sample of  $\beta$ -glucosidase, Endo-Lys C protease was added to 1% of the total protein and the mixture  
5 incubated for 1 hour at 37°C or the protein sample was subject to cyanogen bromide treatment. An equal volume of HPLC solution A (0.05% TEA/0.05% TFA in water) was added to stop the reaction. The resulting CNBr and Endo-Lys C fragments were separated by chromatography  
10 on a Brownlee C-4 column using a linear gradient of 0-100% HPLC solution B (0.05% TEA/0.05% TFA in n-propanol) at a rate of 1% per minute. Several peaks were collected for amino acid sequencing and the data are denoted in Fig. 1.

15

#### Example 9

##### Identification of bgl1 gene from T. reesei

The 700 bp bgl1 cDNA fragment was then labelled with  $^{32}\text{P}$  using methods described by Sambrook et al, supra.

20

Genomic DNA from T. reesei was prepared by filtering a 24-36 hour culture of T. reesei through Miracloth and freezing the mycelia obtained from the culture medium. The frozen mycelia were then ground into a fine powder and 22 mls of TE, and 4 mls of 20%  
25 SDS were added to the powdered mycelia and mixed. 10 ml of phenol and chloroform was added to the mixture prior to centrifugation and removal of the aqueous phase. 200  $\mu\text{l}$  of 5 mg/ml proteinase K was added to the organic extract, and the mixture was incubated for 20 minutes  
30 at 55°C. The DNA was then further extracted by methods

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known in the art using chloroform/phenol extraction followed by ethanol precipitation. The isolated DNA was then treated with 1  $\mu$ g of heated ribonuclease A (100°C for 15 minutes) per 20  $\mu$ g of genomic DNA in TE  
5 buffer at 37°C for 30 minutes, then cooled to room temperature. The genomic DNA from T. reesei was then cut singly or in combination with a variety of restriction enzymes such as Eco RI, Hind III and the like, Southern blotted and hybridized with the P<sup>32</sup>  
10 labelled 700 bp cDNA fragment of the bgl1 gene as a probe. From this analysis it was determined that Hind III was the restriction enzyme of choice used to locate the  $\beta$ -glucosidase gene.

10 to 20 units of Hind III per milligram of genomic  
15 DNA was added to the DNA and then the DNA was extracted with phenol-chloroform to remove protein. The treated DNA was then alcohol precipitation and resuspended to 2 grams/liter in TE buffer.

4  $\mu$ l samples from the Hind III digestion of genomic  
20 DNA were loaded on a 1% agarose gel and fractionated electrophoretically. The gel was then Southern blotted and probed with the P<sup>32</sup> 700 bp cDNA probe. A 6.0 kb band was identified on the Southern blot of Hind III digested genomic DNA from T. reesei.

25 The remaining Hind III genomic DNA was then subjected to a preparative gel electrophoresis and fragments ranging from 5 kb to 7 kb were then electroeluted from the agarose gel and cloned into Hind  
III digested pUC218. The resulting plasmids were used  
30 to transform E. coli JM101 to create a library. Then the library was screened by colony hybridization using

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p<sup>32</sup> labelled 700 bp bgl1 cDNA as a probe to identify those colonies containing DNA coding for the bgl1 gene.

The positive colonies from the transformation were then picked and the DNA isolated therefrom by  
5 phenol:chloroform extraction and ethanol precipitation, described by Sambrook et al, supra.

The isolated DNA from the positive colonies was digested both singly and in various combinations with the following restriction enzymes: Hind III, Eco RI,  
10 Sst I, Kpn I, Bam HI, Xho I, Bgl II, Cla I, Xba I, Sal I, Pst I, Sph I, Bal I, and Pvu II. The digestions were subjected to agarose gel electrophoresis, and the resultant banding pattern was used to construct a restriction map of the cloned 6.0 kb genomic DNA. The  
15 same agarose gel was Southern blotted and probed with the p<sup>32</sup> labelled 700 bp bgl1 cDNA to identify which genomic restriction fragments shared homology with the bgl1 cDNA. The mapping experiments confirmed that the entire bgl1 gene is contained on the genomic Hind III  
20 clone.. Pvu II and Bal I restriction fragments which ranged in size from 600 bp to 1500 bp hybridized with the 700 bp DNA bgl1 clone and were chosen for subcloning into pUC218 phagemid. After cloning these fragments into the phagemid, the Pvu II and Bal I  
25 subclones were then sequenced using the dideoxy chain termination method of Sanger et al (1977). It was then determined from this sequencing that the overlapping sequences of the subclones aligned with a single contiguous sequence totaling 3033 bp within which the  
30 nucleotide sequence was determined on both strands.

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Example 10Construction of pSASB-glu

The starting vector for the construction of pSASB-glu was the plasmid pSAS. pSAS was constructed in the following way. pUC100 (a commercially available plasmid vector) was digested with the restriction enzyme SmaI and the 5' phosphate groups subsequently removed by digestion with calf intestinal alkaline phosphatase. The linear vector fragment was purified from undigested vector and protein by agarose gel electrophoresis followed by isolation of the linear vector DNA from the isolated gel slice by electro-elution. The amdS gene was isolated as a 2.4 kb SstI restriction fragment following separation from the vector sequences (contained in - Hynes, M.J., Corrick, C.M., and King, J.A., "Isolation of genomic clones containing the amdS gene of Aspergillus nidulans and their use in the analysis of structural and regulatory mutations", Mol. Cell. Biol., Vol. 3 (1983), pp. 1430-1439). The 2.4 kb SstI amdS fragment and the 2.7 kb pUC100 vector fragment were then ligated together (Sambrook et al., supra) and the ligation mix transformed and propagated in the E. coli host strain, JM101.

pSASB-glu was constructed by digesting pSAS with the restriction enzyme Hind III, and purifying the linear fragment as described above. Into this Hind III treated pSAS vector fragment was ligated a 6.0 kb Hind III fragment of T. reesei genomic DNA that contained all of the coding region of the bgl1 gene along with sequences necessary for the genes transcription and translation.

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Example 11Preparation of BGL1 Deletion Vector

The gene replacement vector pUC $\Delta$  $\beta$ -Glu A/R pyr, illustrated in Figure 3B, was constructed by cloning a 6.0 kb genomic HindIII fragment, known to contain the entire bgl1 gene, into the polylinker of pUC218 which had been cut with HindIII and the ends dephosphorylated with calf intestinal alkaline phosphatase. The coding region for the bgl1 gene was then removed from this plasmid by digesting the plasmid with ApaI and EcoRV at unique ApaI and EcoRV restriction sites situated at the very 5' and 3' end of the bgl1 open reading frame and isolating the linear plasmid DNA. The restriction site ends were made blunt with T4 DNA polymerase. This plasmid was then ligated with an isolated 2412 bp HindIII/Bam HI restriction fragment containing the pyrG gene from Aspergillus niger (Hartingsreldt et al., Mol. Gen. Genet. 206:71-75 (1987) in which the restriction ends were made blunt by treatment with T4 DNA polymerase to create pUC $\Delta$  $\beta$ Glu A/R pyr (Fig. 3B).

Example 12Isolation of Protoplasts

Mycelium was obtained by inoculating 100 ml of YEG (0.5% yeast extract, 2% glucose) in a 500 ml flask with about  $5 \times 10^7$  T. reesei cells. The flask was then incubated at 37°C with shaking for about 16 hours. The mycelium was harvested by centrifugation at 2,750 x g. The harvested mycelium were further washed in 1.2 M sorbitol solution and resuspended in 40 ml of Novozym<sup>R</sup>, which is the tradename for a multi-component enzyme

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system containing 1,3-alpha-glucanase, 1,3-beta-glucanase, laminarinase, xylanase, chitinase and protease from Novo Biolabs, Danbury, Ct., solution containing 5 mg/ml Novozym<sup>R</sup> 234; 5 mg/ml MgSO<sub>4</sub>·7H<sub>2</sub>O; 0.5  
5 mg/ml bovine serum albumin; 1.2 M sorbitol. The protoplasts were removed from cellular debris by filtration through Miracloth (Calbiochem Corp.) and collected by centrifugation at 2,000 x g. The protoplasts were washed three times in 1.2 M sorbitol and once in 1.2 M  
10 sorbitol, 50 mM CaCl<sub>2</sub>, centrifuged and resuspended. The protoplasts were finally resuspended at a density of 2 x 10<sup>8</sup> protoplasts per ml of 1.2 M sorbitol, 50 mM CaCl<sub>2</sub>.

#### Example 13

##### Transformation of Fungal Protoplasts with pSASβ-glu

15 200 μl of the protoplast suspension prepared in Example 12 was added to 20 μl (20 μg) of pSASβ-glu in TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA) and 50 μl of a polyethylene glycol (PEG) solution containing 25% PEG  
20 4000, 0.6 M KCl and 50 mM CaCl<sub>2</sub>. This mixture was incubated on ice for 20 minutes. After this incubation period 2.0 ml of the above-identified PEG solution was added thereto, the solution was further mixed and incubated at room temperature for 5 minutes. After  
25 this second incubation, 4.0 ml of a solution containing 1.2 M sorbitol and 50 mM CaCl<sub>2</sub> was added thereto and this solution was further mixed. The protoplast solution was then immediately added to molten aliquot's  
of Vogels Medium N (3 grams sodium citrate, 5 grams KH<sub>2</sub>PO<sub>4</sub>, 2 grams NH<sub>4</sub>NO<sub>3</sub>, 0.2 grams MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 gram  
30 CaCl<sub>2</sub>·2H<sub>2</sub>O, 5 μg α-biotin, 5 mg citric acid, 5 mg

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ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1 mg Fe(NH<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.25 mg CuSO<sub>4</sub>·5H<sub>2</sub>O, 50 µg MnSO<sub>4</sub>·4H containing an additional 1% glucose, 1.2 M sorbitol and 1% agarose. The protoplast/medium mixture was then poured onto a solid medium containing the same Vogel's medium as stated above containing in addition acetamide as a nitrogen source. Since T. reesei does not contain a functional equivalent to the amdS gene only transformants will grow on this medium. These colonies were subsequently transferred and purified on a solid Vogel's medium N containing as an additive, 1% glucose. The bgl1 gene inserted transformant strain is called A83pSASBGlu.

Stable transformants can be distinguished from unstable transformants by their faster growth rate and the formation of circular colonies with a smooth rather than ragged outline on solid culture medium. Additionally, in some cases, a further test of stability can be made by growing the transformants on solid non-selective medium, harvesting the spores from this culture medium and determining the percentage of these spores which will subsequently germinate and grow on selective medium.

Figure 6 is an autoradiograph of a Southern blot using the P<sup>32</sup> labelled 700 bp fragment as a probe, of the different transformants with enhanced copies of the bgl1 gene (lanes 1-8) using genomic T. reesei from an overproducing strain digested with Hind III as a control (lane 9). This autoradiograph clearly shows that the transformants contained enhanced amount of the bgl1 gene compared with the control.

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Figure 4 is an autoradiograph of a Northern blot of RNA isolated from one of the transformed strains (lane A) produced by the present invention following induction with sorbose illustrating a corresponding increase in the levels of bgl1 message when compared to the parental strain of T. reesei (lane B).

Besides visual analysis of the transformants, quantitative analysis was also completed by cutting the appropriate bands out of the Nytran<sup>R</sup> membrane and counting the radioactive label present therein in a scintillation counter. This experiment was performed to obtain a more precise estimate of the relative amounts of message as shown in Table III below:

TABLE III

CPM	Parental <u>Trichoderma reesei</u> strain	Transformed <u>Trichoderma reesei</u> strain
CPM $\beta$ -glu message	14.4	25.4
CPM CBHII	227.1	95.2
CPM $\beta$ -glu/ CBHII	0.0634	0.2668

Table III illustrates that the transformant produced by the process of the present invention has extra  $\beta$ -glucosidase mRNA and hence an increase in  $\beta$ -glucosidase enzyme resulting in an increase in specific activity.

Example 14

Transformation of Fungal Protoplasts with pUCA $\beta$ Glu A/R pyr4



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Mutants of T. reesei lacking the coding sequence for the extracellular  $\beta$ -glucosidase gene, bglI, were obtained by a targeted gene replacement event. pUC $\Delta$  $\beta$ Glu A/R pyr4 plasmid was digested with Hind III to obtain a linear HindIII fragment in which the bglI coding sequences were replaced with the pyrG gene from Aspergillus niger. Protoplasts were transformed with the linear DNA fragment containing the bgl1 flanking sequences and the pyr4 by the methods of Examples 12 and 13. The deletion transformants were called  $\Delta$ 12 and  $\Delta$ 36. After transformation, the protoplast solution was then added to molten aliquots of Vogel's Medium N containing an additional 1% glucose, 1.2 M sorbitol and 1% agarose. The protoplast/medium mixture was then poured into a solid medium containing the same Vogel's medium N. No uridine was present in the medium and therefore only transformed colonies were able to grow as a result of complementation of the pyr4 mutation of the T. reesei strain RL-P37 by the wild type pyr4 gene inserted in the DNA fragment. Stable transformants were then selected by the method recited in Example 13.

#### Example 15

##### Analysis of the Transformants

The transformants were analyzed for the presence or absence of the bgl1 gene using the 700 bp cDNA probe recited above. The transformants were digested using HindIII. Total genomic DNA from selected transformants was digested with HindIII restriction enzyme, run on a 1% agarose gel, transferred to Nitran<sup>R</sup> membrane and probed with a P<sup>32</sup> labelled 700 bp cDNA rectied above and

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visualized by autoradiography on X-ray film. The results of this analysis are set forth in Figure 5A illustrate that the transformants ( $\Delta 12$  and  $\Delta 36$ ) did not contain a band corresponding to the bgl1 gene whereas the wild type strain (RL-P37, i.e., P-37) did.

5 mRNA isolated from the transformants of Example 14 and analyzed on a Northern blot, as in Example 2. As indicated in Figure 5B, Northern blot analysis using the  $P^{32}$  labelled 2.2 Kb ApaI/EcoRV bgl1 probe indicated that bgl1 specific mRNA was present in T. reesei RL-P37 pyrG69 and is absent in the transformants  $\Delta 12$  and  $\Delta 36$ .

Protein was recovered as per Example 8 above and then analyzed for the presence of  $\beta$ -glucosidase by use of polyclonal antibodies (from rabbits challenged with pure  $\beta$ -glucosidase) tagged with horseradish peroxidase to permit detection. The antibodies were used to identify pure  $\beta$ -glucosidase (100 ng -- Column A; 1000 ng -- Column B); cellulase produced from wild type T. reesei (Column C); and from cellulase produced by a T. reesei strain genetically engineered to delete the  $\beta$ -glucosidase gene (Column D). The results of this analysis are set forth in Figure 5C and show that only Column D did not contain  $\beta$ -glucosidase.

While the invention has been described in terms of various preferred embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions, and changes may be made without departing from the scope thereof. Accordingly, it is intended that the scope of the present invention be limited solely by the scope of the following claims, including equivalents thereof.

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**WHAT IS CLAIMED IS:**

1           1. A process for expressing enhanced extracellular  
2      $\beta$ -glucosidase in a filamentous fungus comprising  
3     expressing a fungal DNA sequence encoding enhanced  
4      $\beta$ -glucosidase in a recombinant host microorganism, said  
5     recombinant host microorganism being a filamentous  
6     fungus transformed with an expression vector containing  
7     said DNA sequence.

1           2. The process according to Claim 1, wherein said  
2     expression vector comprises at least one additional  
3     copy of a fungal  $\beta$ -glucosidase gene encoding for  
4     extracellular  $\beta$ -glucosidase expression.

1           3. The process according to Claim 1, further  
2     comprising the step of isolating transformants having  
3     enhanced  $\beta$ -glucosidase expression.

1           4. The process according to Claim 3, further  
2     comprising the steps of:  
3         (a) culturing said transformants under conditions  
4     to permit growth of said transformants; and  
5         (b) isolating a recombinant fungal cellulase  
6     composition produced from said transformants.

1           5. The process according to Claim 4, further  
2     comprising the step of purifying  $\beta$ -glucosidase from  
3     said isolated recombinant fungal cellulase composition.

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1           6. The process according to Claim 1, wherein said  
2 filamentous fungus is selected from the genus of  
3 Trichoderma, Aspergillus, Neurospora, Humicola and  
4 Penicillium.

1           7. The process according to Claim 6, wherein said  
2 filamentous fungus is Trichoderma reesei, Trichoderma  
3 viridae, Trichoderma koningii, Aspergillus niger,  
4 Aspergillus nidulans, Aspergillus wentii, Aspergillus  
5 oryzae, Aspergillus phoenicis, Neurospora crassa,  
6 Humicola grisea, Penicillium pinophilum or Penicillium  
7 oxalicum.

1           8. The process according to Claim 2, wherein said  
2 expression vector comprises all of a coding region of a  
3 fungal  $\beta$ -glucosidase gene and sequences necessary for  
4 the  $\beta$ -glucosidase gene's transcription and translation.

1           9. The process according to Claim 8, wherein said  
2  $\beta$ -glucosidase gene is a bgl1 gene derived from  
3 Trichoderma reesei.

1           10. The process according to Claim 9, wherein said  
2 bgl1 gene substantially comprises the following amino  
3 acid and nucleotide sequence from 311 to 2679 of  
4 Figure 1.

1           11. The process according to Claim 4, wherein said  
2 recombinant fungal cellulase composition is isolated  
3 by:

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- 4 (a) centrifuging said culture medium containing  
5 said transformants having enhanced  $\beta$ -glucosidase  
6 activity to form a supernatant and a pellet; and  
7 (b) filtering said supernatant to obtain a  
8 recombinant fungal cellulase composition.

1 12. The process according to Claim 11, wherein an  
2 antimicrobial agent is added to said recombinant fungal  
3 cellulase composition after filtration.

1 13. Transformants having enhanced extracellular  
2  $\beta$ -glucosidase expression produced by the process  
3 according to Claim 3.

1 14. A fungal cellulase composition derived from a  
2 filamentous fungus which is enriched by recombinant  
3 means in  $\beta$ -glucosidase.

1 15. The fungal cellulase composition according to  
2 Claim 14, wherein said filamentous fungus is selected  
3 from the genus of Trichoderma, Aspergillus, Neurospora,  
4 Humicola, and Penicillium.

1 16. The fungal cellulase composition according to  
2 Claim 14, wherein said filamentous fungus is  
3 Trichoderma reesei, Trichoderma viridae, Trichoderma  
4 koningii, Aspergillus niger, Aspergillus nidulans,  
5 Aspergillus wentii, Aspergillus oryzae, Aspergillus  
6 phoenicis, Neurospora crassa, Humicola grisea,  
7 Penicillium pinophilum, Penicillium oxalicum, or  
8 admixtures thereof.

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1           17. A process for expressing cellulases but does  
2 not express extracellular  $\beta$ -glucosidase in a  
3 filamentous fungus comprising expressing a fungal DNA  
4 sequence encoding cellulases but not encoding  $\beta$ -gluco-  
5 sidase in a recombinant host microorganism, said  
6 recombinant host microorganism being a filamentous  
7 fungus transformed with an expression vector containing  
8 said DNA sequence.

1           18. The process according to Claim 17, wherein  
2 said expression vector comprises genes encoding for  
3 cellulase expression but does not contain a  $\beta$ -gluco-  
4 sidase gene encoding for  $\beta$ -glucosidase expression.

1           19. The process according to Claim 17, further  
2 comprising the step of isolating transformants which  
3 are able to express other cellulases but do not express  
4  $\beta$ -glucosidase.

1           20. The process according to Claim 19, further  
2 comprising the steps of:  
3           (a) culturing said transformants under conditions  
4 to permit growth of said transformants; and  
5           (b) isolating a recombinant fungal cellulase  
6 composition produced from said transformants.

1           21. The process according to Claim 20, further  
2 comprising the step of purifying cellulases from said  
3 isolated recombinant fungal cellulase composition.

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1           22. The process according to Claim 17, wherein  
2           said filamentous fungus is selected from the genus of  
3           Trichoderma, Aspergillus, Neurospora, Humicola, and  
4           Penicillium.

1           23. The process according to Claim 22, wherein  
2           said filamentous fungus is Trichoderma reesei,  
3           Trichoderma viridae, Trichoderma koningii, Aspergillus  
4           niger, Aspergillus nidulans, Aspergillus wentii,  
5           Aspergillus oryzae, Aspergillus phoenicis, Neurospora  
6           crassa, Humicola grisea, Penicillium pinophilum or  
7           Penicillium oxalicum.

1           24. The process according to Claim 18, wherein  
2           said deleted  $\beta$ -glucosidase gene is a bgl1 gene derived  
3           from Trichoderma reesei.

1           25. The process according to Claim 21, wherein  
2           said deleted bgl1 gene substantially comprises the  
3           following amino acid and nucleotide sequence from base  
4           numbers 311 to 2679 of Figure 1.

1           26. The process according to Claim 20, wherein  
2           said recombinant fungal cellulase composition is  
3           isolated by:

4           (a) centrifuging said culture medium containing  
5           said transformants containing cellulase expression but  
6           do not express  $\beta$ -glucosidase to form a supernatant and  
7           a pellet; and

8           (b) filtering said supernatant to obtain a  
9           recombinant fungal cellulase composition.

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1           27. The process according to Claim 26, wherein an  
2 antimicrobial agent is added to said recombinant fungal  
3 cellulase composition after filtration.

1           28. Transformants which express cellulases but do  
2 not express  $\beta$ -glucosidase produced by the process  
3 according to Claim 19.

1           29. A fungal cellulase composition derived from a  
2 filamentous fungus which comprises by recombinant means  
3 all cellulases except  $\beta$ -glucosidase.

1           30. The fungal cellulase composition according to  
2 Claim 29, wherein said filamentous fungus is selected  
3 from the genus of Trichoderma, Aspergillus, Neurospora,  
4 Humicola, and Penicillium.

1           31. The fungal cellulase composition according to  
2 Claim 30, wherein said filamentous fungus is  
3 Trichoderma reesei, Trichoderma viridae, Trichoderma  
4 koningii, Aspergillus niger, Aspergillus nidulans,  
5 Aspergillus wentii, Aspergillus oryzae, Aspergillus  
6 phoenicis, Neurospora crassa, Humicola grisea,  
7 Penicillium pinophilum, Penicillium oxalicum, or  
8 admixtures thereof.

1           32. A process for expressing an altered extra-  
2 cellular  $\beta$ -glucosidase in a filamentous fungus  
3 comprising expressing a fungal DNA sequence encoding an  
4 altered  $\beta$ -glucosidase in a recombinant host  
5 microorganism, said recombinant host microorganism



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6 being a filamentous fungus transformed with an  
7 expression vector containing said DNA sequence.

1 33. The process according to Claim 32, wherein  
2 said expression vector comprises an altered fungal  
3  $\beta$ -glucosidase gene encoding for altered extracellular  
4  $\beta$ -glucosidase.

1 34. The process according to Claim 32, further  
2 comprising the step of isolating transformants having  
3 expression of altered  $\beta$ -glucosidase.

1 35. The process according to Claim 34, further  
2 comprising the steps of:  
3 (a) culturing transformants under conditions to  
4 permit growth of said transformants; and  
5 (b) isolating a recombinant fungal cellulase  
6 composition produced from said transformants.

1 36. The process according to Claim 35, further  
2 comprising the step of purifying said altered fungal  
3  $\beta$ -glucosidase from said isolated recombinant fungal  
4 cellulase composition.

1 37. The process according to Claim 32, wherein  
2 said filamentous fungus is selected from the genus of  
3 Trichoderma, Aspergillus, Neurospora, Humicola, and  
4 Penicillium.

1 38. The process according to Claim 37, wherein  
2 said filamentous fungus is Trichoderma reesei,

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3 Trichoderma viridae, Trichoderma koningii, Aspergillus  
4 niger, Aspergillus nidulans, Aspergillus wentii,  
5 Aspergillus oryzae, Aspergillus phoenicis, Neurospora  
6 crassa, Humicola grisea, Penicillium pinophilum or  
7 Penicillium oxalicum.

1 39. The process according to Claim 32, wherein  
2 said altered fungal  $\beta$ -glucosidase gene is a bgl1 gene  
3 derived from Trichoderma reesei.

1 40. The process according to Claim 39, wherein the  
2 bgl1 gene amino acid and nucleotide sequence of Figure  
3 1 can be altered.

1 41. The process according to Claim 34, wherein  
2 said recombinant fungal cellulase composition is  
3 isolated by:

4 (a) centrifuging said culture medium containing  
5 transformants having said altered  $\beta$ -glucosidase  
6 expression to form a supernatant and a pellet; and

7 (b) filtering said supernatant to obtain a  
8 recombinant fungal cellulase composition.

1 42. The process according to Claim 41, wherein an  
2 antimicrobial agent is added to said recombinant fungal  
3 cellulase composition after filtration.

1 43. Transformants expressing said altered fungal  
2  $\beta$ -glucosidase produced by the process according to  
3 Claim 34.

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1           44. A fungal cellulase composition derived from a  
2 filamentous fungus which is an altered  $\beta$ -glucosidase  
3 produced by recombinant means.

1           45. The fungal cellulase composition according to  
2 Claim 44, wherein said filamentous fungus is selected  
3 from the genus of Trichoderma, Aspergillus, Neurospora,  
4 Humicola, and Penicillium.

1           46. The fungal cellulase composition according to  
2 Claim 45, wherein said filamentous fungus is  
3 Trichoderma reesei, Trichoderma viridae, Trichoderma  
4 koningii, Aspergillus niger, Aspergillus nidulans,  
5 Aspergillus wentii, Aspergillus oryzae, Aspergillus  
6 phoenicis, Neurospora crassa, Humicola grisea,  
7 Penicillium pinophilum, Penicillium oxalicum, or  
8 admixtures thereof.

1           47. A method for enhancing the degradation of  
2 cellulose to glucose by using a recombinant fungal  
3 cellulase composition derived from a filamentous fungus  
4 which has enhanced  $\beta$ -glucosidase activity.

1           48. The method of use according to Claim 47,  
2 wherein said filamentous fungus is selected from the  
3 genus of Trichoderma, Aspergillus, Neurospora,  
4 Humicola, and Penicillium.

1           49. The method of use according to Claim 48,  
2 wherein said filamentous fungus is Trichoderma reesei,  
3 Trichoderma viridae, Trichoderma koningii, Aspergillus

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4 niger, Aspergillus nidulans, Aspergillus wentii,  
5 Aspergillus oryzae, Aspergillus phoenicis, Neurospora  
6 crassa, Humicola grisea, Penicillium pinophilum,  
7 Penicillium oxalicum, or admixtures thereof.

1 50. A composition that increases the rate and  
2 extent of production of glucose from cellulosics or  
3 hetero-glycans, said composition comprising a  
4 recombinant fungal cellulase composition having  
5 enhanced  $\beta$ -glucosidase activity.

1 51. The composition according to Claim 50, wherein  
2 said recombinant fungal cellulase composition having  
3 enhanced  $\beta$ -glucosidase activity may be used to degrade  
4 cellulosics present in feedstock, biomass and sludge.

1 52. A method of using the composition of Claim 50,  
2 said method comprising adding said recombinant fungal  
3 cellulase composition to yeast and biomass to form a  
4 mixture and culturing said mixture at a sufficient  
5 temperature and for a sufficient period of time to  
6 produce ethanol.

1 53. A method of using the transformants of  
2 Claim 13, said method comprising adding said  
3 transformants to yeast and biomass to form a mixture  
4 and culturing said mixture at a sufficient temperature  
5 and for a sufficient period of time to produce ethanol.

1 54. The method according to Claims 52 or 53,  
2 wherein said yeast is B. clausenii, S. cerevisiae,

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3     Celluloyticus acidothermophilum, C. brassicae, C.  
4     lustinaniae, S. uvarum or Schizosaccharomyces pombe.

1           55. A fungal cellulase composition containing  
2     enhanced extracellular fungal  $\beta$ -glucosidase activity  
3     comprising at least one additional extracellular  $\beta$ -  
4     glucosidase wherein said extracellular  $\beta$ -glucosidase  
5     substantially comprises the amino acid and nucleotide  
6     sequence from base numbers 311 to 2679 of Figure 1.

1           56. A method of using purified  $\beta$ -glucosidase  
2     produced from the process according to Claim 5 in food  
3     additives to enhance the flavor and aroma of foods.

1           57. A method of using the recombinant fungal  
2     cellulase composition produced according to Claim 14 in  
3     food additives to enhance the flavor and aroma.

1           58. A detergent composition comprising a cleaning  
2     effective amount of a surfactant and at least .0001  
3     weight percent of a recombinant fungal cellulase  
4     composition according to Claim 14.

1           59. A detergent composition comprising a cleaning  
2     effective amount of a surfactant and at least .0001  
3     weight percent of a recombinant fungal cellulase  
4     composition according to Claim 29.

1           60. A detergent composition comprising a cleaning  
2     effective amount of a surfactant and at least .0001

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3 weight percent of a recombinant fungal cellulase  
4 composition according to Claim 44.

1 61. A nucleotide sequence of a bgl1 gene which  
2 entire sequence or a portion can be labeled and used as  
3 a probe wherein the bgl1 gene has the nucleotide  
4 sequence of Figure 1.

1 62. A method of using the probe according to  
2 Claim 61, for identifying a  $\beta$ -glucosidase gene from a  
3 filamentous fungus.

1 63. The method according to Claim 62, wherein said  
2 probe is derived from Trichoderma reesei.

1 64. The method according to Claim 62, wherein one  
2 or more probes can be used to identify said  $\beta$ -  
3 glucosidase gene from a filamentous fungus.

1 65. A method for producing cellooligosaccharides  
2 which comprises contacting cellulose containing  
3 materials with a cellulase composition free of  
4  $\beta$ -glucosidase at a pH of from about 4.5 to about 8.

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**FIG. 1**

TGGCACAGA GGGAGAGTTC GGGCTAAGGC TTGGTGGAGG AAATGATGG CCAAGGCGTC	60
AAATGGTAAA TCTGGGTGTG GGTAGGAGTG CAAAGATGGG ATTTGGGCGC AATGCTGCGG	120
AGGCGAGTG TTTCTGCAAC GTTATOCAGG AGATTGGCGC TTGGOCAAGA GGGAGTTGAC	180
GGGAGAGTTC CCAACTGGTT CCTTCAGTAA CGGCAAGCTG GCAGACTATA TAACTTGTGG	240
ACAAGACTCT GCTTTGTGA GTTCTTCTA CCACTCTGA CCAAGACCAT TCTGTTGAGC	300
CCAATCAGAA ATG CGT TAC CGA ACA GCA GCT GCG CTG GCA CTT GGC ACT	349
Met Arg Tyr Arg Thr Ala Ala Ala Leu Ala Leu Ala Thr	
1 5 10	
GGG CCG TTT GCT AGG GCA GAC AGT CA GTATAGCTGG TCCATACTGG	395
Gly Pro Phe Ala Arg Ala Asp Ser His	
15 20	

**FIG. 1 (Con't.)**

GATGTGATAT GTATCTGGA GACAOCATGC TGACTCTTGA ATCAAGGTAG C TCA ACA	452
Ser Thr	
TOG GGG GOC TOG GCT GAG GCA GTT GTA OCT OCT GCA GGG ACT OCA TGG	500
Ser Gly Ala Ser Ala Glu Ala Val Val Pro Pro Ala Gly Thr Pro Trp	
25 30 35 40	
GGA AOC GOG TAC GAC AAG GOG AAG GOC GCA TTG GCA AAG CTC AAT CTC	548
Gly Thr Ala Tyr Asp Lys Ala Lys Ala Ala Leu Ala Lys Leu Asn Leu	
45 50 55	
CAA GAT AAG GTC GGC ATC GTG AGC GGT GTC GGC TGG AAC GGC GGT OCT	596
Gln Asp Lys Val Gly Ile Val Ser Gly Val Gly Trp Asn Gly Gly Pro	
60 65 70	
TGC GTT GGA AAC ACA TCT OCG GOC TOC AAG ATC AGC TAT OCA TCG CTA	644
Cys Val Gly Asn Thr Ser Pro Ala Ser Lys Ile Ser Tyr Pro Ser Leu	
75 80 85	
TGC CTT CAA GAC GGA OCG CTC GGT GTT OGA TAC TOG ACA GGC AGC ACA	692
Cys Leu Gln Asp Gly Pro Leu Gly Val Arg Tyr Ser Thr Gly Ser Thr	
90 95 100	
GOC TTT ACG OCG GGC GTT CAA GOG GOC TOG ACG TGG GAT GTC AAT TTG	740
Ala Phe Thr Pro Gly Val Gln Ala Ala Ser Thr Trp Asp Val Asn Leu	
105 110 115 120	
ATC OCG GAA CGT GGA CAG TTC ATC GGT GAG GAG GTG AAG GOC TOG GGG	788
Ile Arg Glu Arg Gly Gln Phe Ile Gly Glu Glu Val Lys Ala Ser Gly	
125 130 135	
ATT CAT GTC ATA CTT GGT OCT GTG GCT GGG OCG CTG GGA AAG ACT OCG	836
Ile His Val Ile Leu Gly Pro Val Ala Gly Pro Leu Gly Lys Thr Pro	
140 145 150	
CAG GGC GGT OCG AAC TGG GAG GGC TTC GGT GTC GAT OCA TAT CTC ACG	884
Gln Gly Gly Arg Asn Trp Glu Gly Phe Gly Val Asp Pro Tyr Leu Thr	
155 160 165	
GGC ATT GOC ATG GGT CAA AOC ATC AAC GGC ATC CAG TOG GTA GOC GTG	932
Gly Ile Ala Met Gly Gln Thr Ile Asn Gly Ile Gln Ser Val Gly Val	
170 175 180	
CAG GOG ACA GOG AAG CAC TAT ATC CTC AAC GAG CAG GAG CTC AAT OGA	980
Gln Ala Thr Ala Lys His Tyr Ile Leu Asn Glu Gln Glu Leu Asn Arg	
185 190 195 200	
GAA AOC ATT TOG AGC AAC OCA GAT GAC OGA ACT CTC CAT GAG CTG TAT	1028
Glu Thr Ile Ser Ser Asn Pro Asp Asp Arg Thr Leu His Glu Leu Tyr	
205 210 215	



**FIG. 1 (Con't.)**

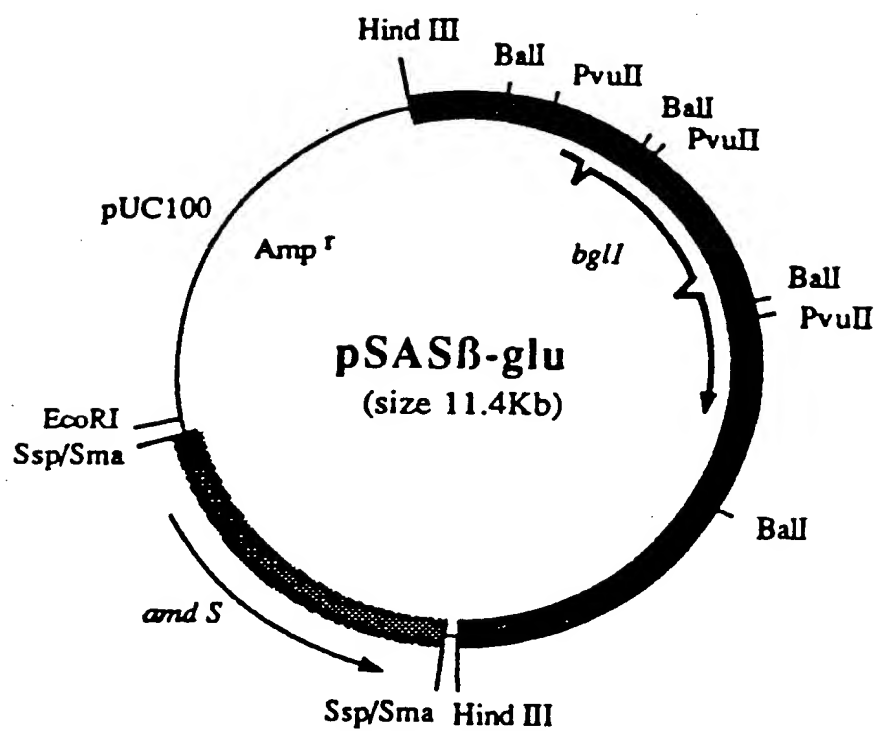
ACT TGG CCA TTT GOC GAC GOG GTT CAG GOC AAT GTC GCT TCT GTC ATG Thr Trp Pro Phe Ala Asp Ala Val Gln Ala Asn Val Ala Ser Val Met 220 225 230	1076
TGC TGG TAC AAC AAG GTC AAT AOC AOC TGG GOC TGC GAG GAT CAG TAC Cys Ser Tyr Asn Lys Val Asn Thr Thr Trp Ala Cys Glu Asp Gln Tyr 235 240 245	1124
ACG CTG CAG ACT GTG CTG AAA GAC CAG CTG GGG TTC CCA GGC TAT GTC Thr Leu Gln Thr Val Leu Lys Asp Gln Leu Gly Phe Pro Gly Tyr Val 250 255 260	1172
ATG ACG GAC TGG AAC GCA CAG CAC ACG ACT GTC CAA AGC GOG AAT TCT Met Thr Asp Trp Asn Ala Gln His Thr Thr Val Gln Ser Ala Asn Ser 265 270 275 280	1220
GGG CTT GAC ATG TCA ATG OCT GGC ACA GAC TTC AAC GGT AAC AAT CCG Gly Leu Asp Met Ser Met Pro Gly Thr Asp Phe Asn Gly Asn Asn Arg 285 290 295	1268
CTC TGG GGT CCA GCT CTC AOC AAT GOG GTA AAT AGC AAT CAG GTC OOC Leu Trp Gly Pro Ala Leu Thr Asn Ala Val Asn Ser Asn Gln Val Pro 300 305 310	1316
ACG AGC AGA GTC GAC GAT ATG GTG ACT CGT ATC CTC GOC GCA TGG TAC Thr Ser Arg Val Asp Asp Met Val Thr Arg Ile Leu Ala Ala Trp Tyr 315 320 325	1364
TTG ACA GGC CAG GAC CAG GCA GGC TAT CCG TGG TTC AAC ATC AGC AGA Leu Thr Gly Gln Asp Gln Ala Gly Tyr Pro Ser Phe Asn Ile Ser Arg 330 335 340	1412
AAT GTT CAA GGA AAC CAC AAG AOC AAT GTC AGG GCA ATT GOC AGG GAC Asn Val Gln Gly Asn His Lys Thr Asn Val Arg Ala Ile Ala Arg Asp 345 350 355 360	1460
GGC ATC GTT CTG CTC AAG AAT GAC GOC AAC ATC CTG CCG CTC AAG AAG Gly Ile Val Leu Leu Lys Asn Asp Ala Asn Ile Leu Pro Leu Lys Lys 365 370 375	1508
CCC GCT AGC ATT GOC GTC GTT GGA TCT GOC GCA ATC ATT GGT AAC CAC Pro Ala Ser Ile Ala Val Val Gly Ser Ala Ala Ile Ile Gly Asn His 380 385 390	1556
GOC AGA AAC TGG OOC TGG TGC AAC GAC AAA GGC TGC GAC GAC GGG GOC Ala Arg Asn Ser Pro Ser Cys Asn Asp Lys Gly Cys Asp Asp Gly Ala 395 400 405	1604
TTG GGC ATG GGT TGG GGT TOC GGC GOC GTC AAC TAT CCG TAC TTC GTC Leu Gly Met Gly Trp Gly Ser Gly Ala Val Asn Tyr Pro Tyr Phe Val 410 415 420	1652

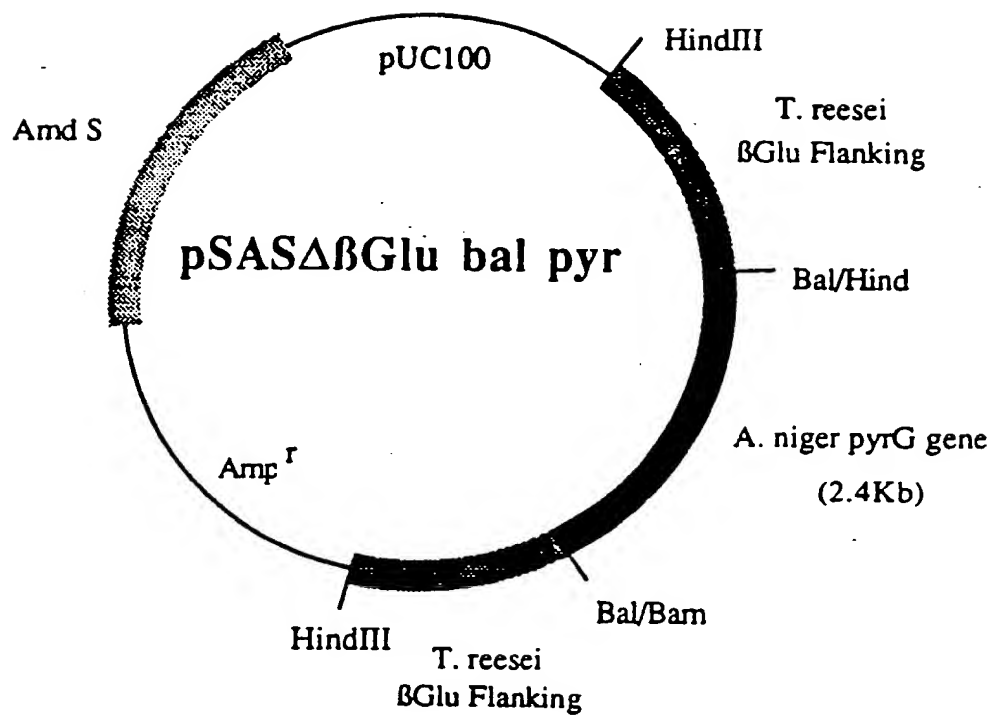
**FIG. 1 (Con't.)**

GCG	CCC	TAC	GAT	GCC	ATC	AAT	ACC	AGA	GCG	TCT	TOG	CAG	GGC	ACC	CAG	1700
Ala	Pro	Tyr	Asp	Ala	Ile	Asn	Thr	Arg	Ala	Ser	Ser	Gln	Gly	Thr	Gln	
425					430				435						440	
GTT	ACC	TTG	AGC	AAC	ACC	GAC	AAC	ACG	TOC	TCA	GGC	GCA	TCT	GCA	GCA	1748
Val	Thr	Leu	Ser	Asn	Thr	Asp	Asn	Thr	Ser	Ser	Gly	Ala	Ser	Ala	Ala	
				445				450						455		
AGA	GGA	AAG	GAC	GTC	GCC	ATC	GTC	TTC	ATC	ACC	GCC	GAC	TOG	GGT	GAA	1796
Arg	Gly	Lys	Asp	Val	Ala	Ile	Val	Phe	Ile	Thr	Ala	Asp	Ser	Gly	Glu	
			460				465						470			
GGC	TAC	ATC	ACC	GTG	GAG	GGC	AAC	GCG	GCC	GAT	GGC	AAC	AAC	CTG	GAT	1844
Gly	Tyr	Ile	Thr	Val	Glu	Gly	Asn	Ala	Gly	Asp	Arg	Asn	Asn	Leu	Asp	
	475					480						485				
CCG	TGG	CAC	AAC	GGC	AAT	GCC	CTG	GTC	CAG	GCG	GTG	GCC	GGT	GCC	AAC	1892
Pro	Trp	His	Asn	Gly	Asn	Ala	Leu	Val	Gln	Ala	Val	Ala	Gly	Ala	Asn	
	490					495					500					
AGC	AAC	GTC	ATT	GTT	GTT	GTC	CAC	TOC	GTT	GGC	GCC	ATC	ATT	CTG	GAG	1940
Ser	Asn	Val	Ile	Val	Val	Val	His	Ser	Val	Gly	Ala	Ile	Ile	Leu	Glu	
505					510				515					520		
CAG	ATT	CTT	GCT	CTT	CCG	CAG	GTC	AAG	GCC	GTT	GTC	TGG	GCG	GGT	CTT	1988
Gln	Ile	Leu	Ala	Leu	Pro	Gln	Val	Lys	Ala	Val	Val	Trp	Ala	Gly	Leu	
			525					530						535		
OCT	TCT	CAG	GAG	AGC	GGC	AAT	GCG	CTC	GTC	GAC	GTG	CTG	TGG	GGA	GAT	2036
Pro	Ser	Gln	Glu	Ser	Gly	Asn	Ala	Leu	Val	Asp	Val	Leu	Trp	Gly	Asp	
		540					545					550				
GTC	AGC	OCT	TCT	GGC	AAG	CTG	GTG	TAC	ACC	ATT	GCG	AAG	AGC	CCC	AAT	2084
Val	Ser	Pro	Ser	Gly	Lys	Leu	Val	Tyr	Thr	Ile	Ala	Lys	Ser	Pro	Asn	
		555				560						565				
GAC	TAT	AAC	ACT	CGC	ATC	GTT	TOC	GGC	GGC	AGT	GAC	AGC	TTC	AGC	GAG	2132
Asp	Tyr	Asn	Thr	Arg	Ile	Val	Ser	Gly	Gly	Ser	Asp	Ser	Phe	Ser	Glu	
	570					575				580						
GGA	CTG	TTC	ATC	GAC	TAT	AAG	CAC	TTC	GAC	GAC	GCC	AAT	ATC	ACG	CCG	2180
Gly	Leu	Phe	Ile	Asp	Tyr	Lys	His	Phe	Asp	Asp	Ala	Asn	Ile	Thr	Pro	
585					590				595					600		
CCG	TAC	GAG	TTC	GGC	TAT	GGA	CTG	T	GTA	GTT	TGC	TAA	CTG	AAC		2225
Arg	Tyr	Glu	Phe	Gly	Tyr	Gly	Leu									
				605												
AATCTATTAG	ACAGGTTGAC	TGA	CGGATGA	CTGTGGAATG	ATAG	CT	TAC	ACC	AAG							2280
								Ser	Tyr	Thr	Lys					
												610				

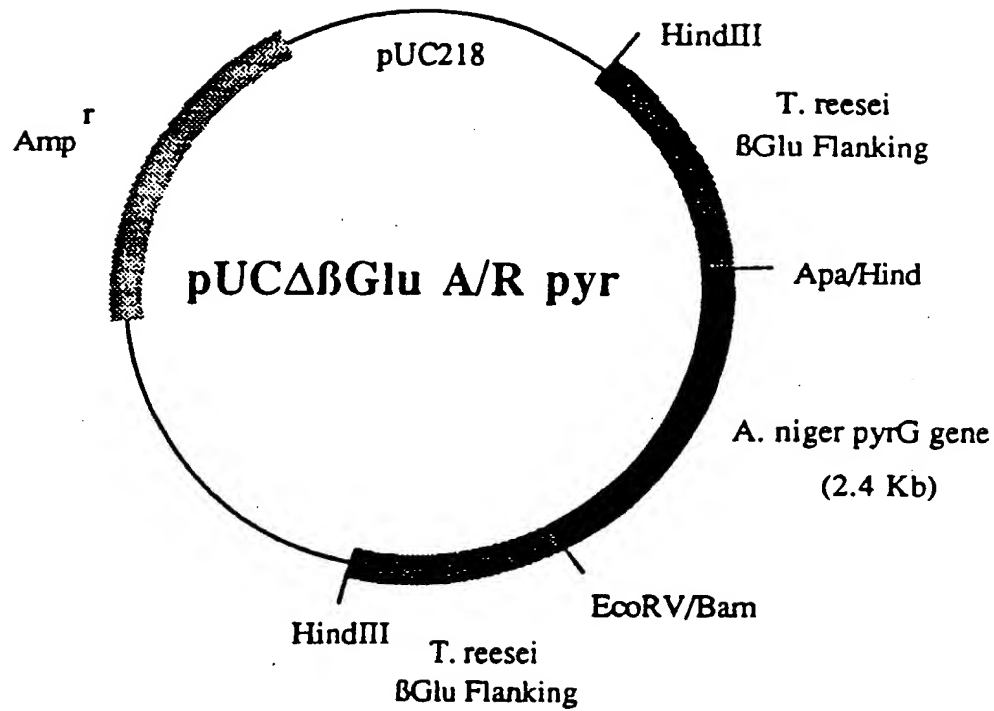
**FIG. 1 (Con't.)**

TTC AAC TAC TCA CCG CTC TOC GTC TTG TOG AOC GOC AAG TCT GGT OCT Phe Asn Tyr Ser Arg Leu Ser Val Leu Ser Thr Ala Lys Ser Gly Pro 615 620 625	2328
GCG ACT GCG GOC GTT GTG OCG GGA GGC OCG AGT GAT CTG TTC CAG AAT Ala Thr Gly Ala Val Val Pro Gly Gly Pro Ser Asp Leu Phe Gln Asn 630 635 640	2376
GTC GCG ACA GTC AOC GTT GAC ATC GCA AAC TCT GGC CAA GTG ACT GGT Val Ala Thr Val Thr Val Asp Ile Ala Asn Ser Gly Gln Val Thr Gly 645 650 655 660	2424
GOC GAG GTA GOC CAG CTG TAC ATC AOC TAC OCA TCT TCA GCA OOC AGG Ala Glu Val Ala Gln Leu Tyr Ile Thr Tyr Pro Ser Ser Ala Pro Arg 665 670 675	2472
AOC OCT OCG AAG CAG CTG OGA GGC TTT GOC AAG CTG AAC CTC ACG OCT Thr Pro Pro Lys Gln Leu Arg Gly Phe Ala Lys Leu Asn Leu Thr Pro 680 685 690	2520
GGT CAG AGC GGA ACA GCA ACG TTC AAC ATC OGA OGA OGA GAT CTC AGC Gly Gln Ser Gly Thr Ala Thr Phe Asn Ile Arg Arg Asp Leu Ser 695 700 705	2568
TAC TGG GAC ACG GCT TOG CAG AAA TGG GTG GTG OCG TOG GCG TOG TTT Tyr Trp Asp Thr Ala Ser Gln Lys Trp Val Val Pro Ser Gly Ser Phe 710 715 720	2616
GOC ATC AGC GTG GGA GCG AGC AGC OCG GAT ATC AGG CTG ACG AGC ACT Gly Ile Ser Val Gly Ala Ser Ser Arg Asp Ile Arg Leu Thr Ser Thr 725 730 735 740	2664
CTG TOG GTA GCG TAGCGGAGG AGGGTGAAGG OGGTTGAOCT GTGACTGTGA Leu Ser Val Ala 745	2716
GTGAGGACCG AAGGTGGAT GGCGTGAATA CTGCAGGAAT ACAATCTTCA GGATAGGCAT	2776
CAGAGCAGTA ACATGAATGA TGAAGACGGC CGAAGCAGAA GTGAATTGAG GAGGTAGTGA	2836
TGATGAAATG TGAGGGAAGA CAGATGTTCA ATCAOCTTGT TOGAGGGAAG CTGCAAATTG	2896
GGOCTCAOCT CATCTGCGAG AGAGAAGGAA CTCTTGCAGC AGGAGTTCTG CTCACTGAGA	2956
AGAAGGGOOG GGTTAGGTC GGGCTCTTC OGGACATOC TOOGCTOOGG CACTGTGCTG	3016
TCAAACCTGGC AOCACAA	3033

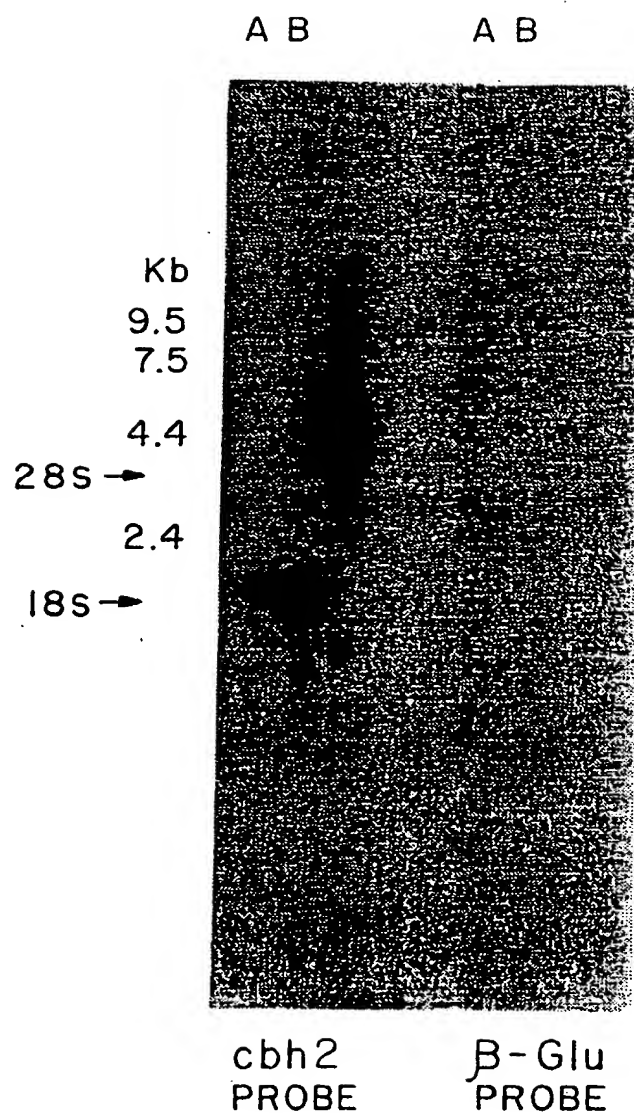
**FIG. 2**

**FIG. 3A**

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**FIG. 3B**

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FIG. 4

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A  
 $\Delta 12$   
 $\Delta 36$   
 P37



FIG. 5A

B  
 P37  
 $\Delta 36$   
 $\Delta 12$

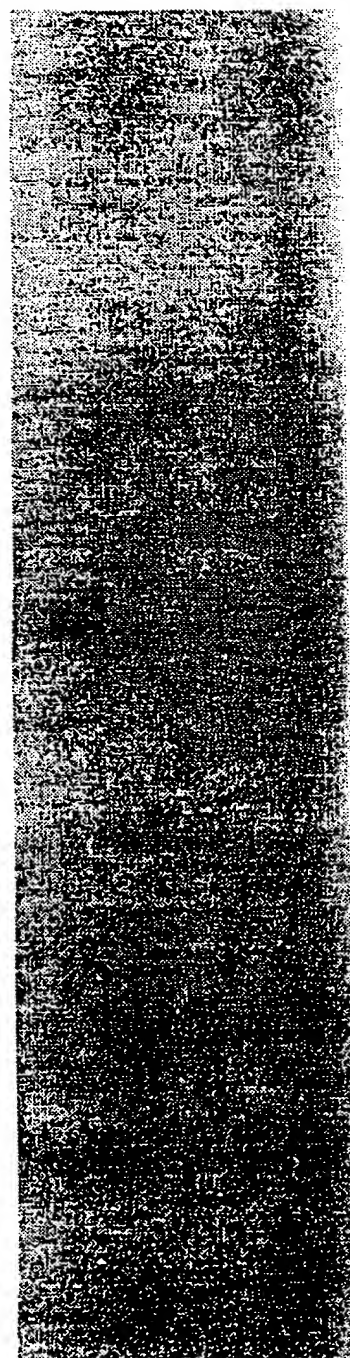


FIG. 5B

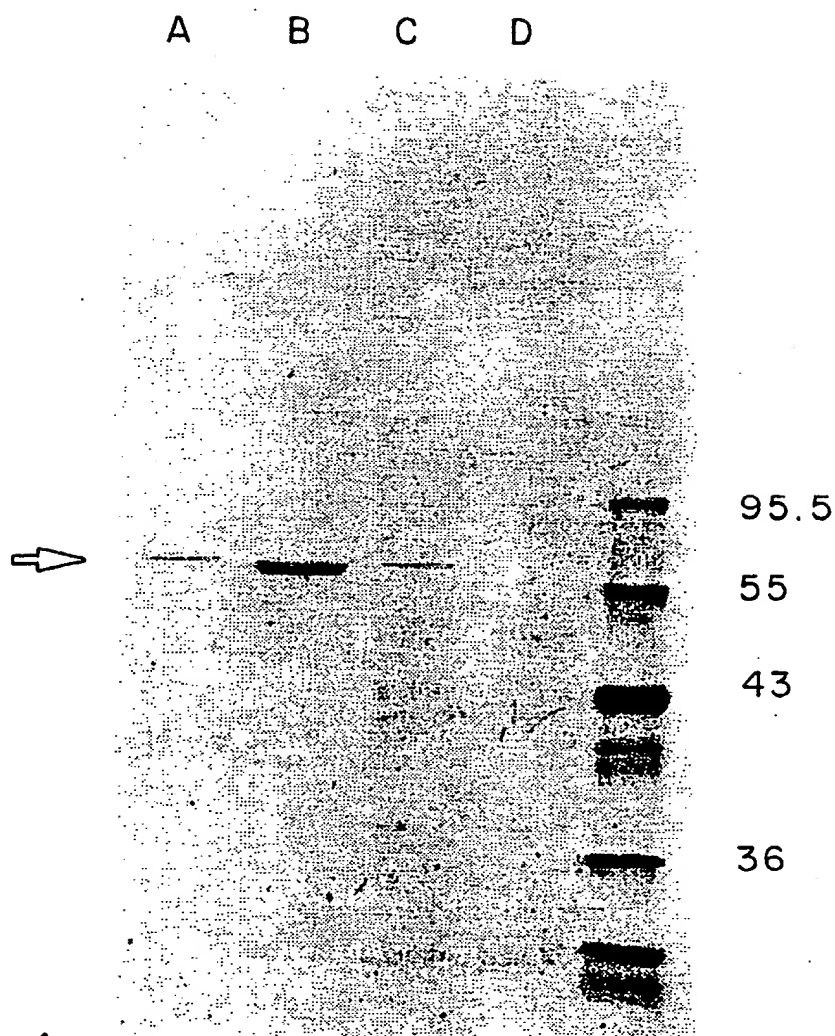
6.0Kb

2.4Kb

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FIG. 5C

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1 2 3 4 5 7 8 9



FIG. 6

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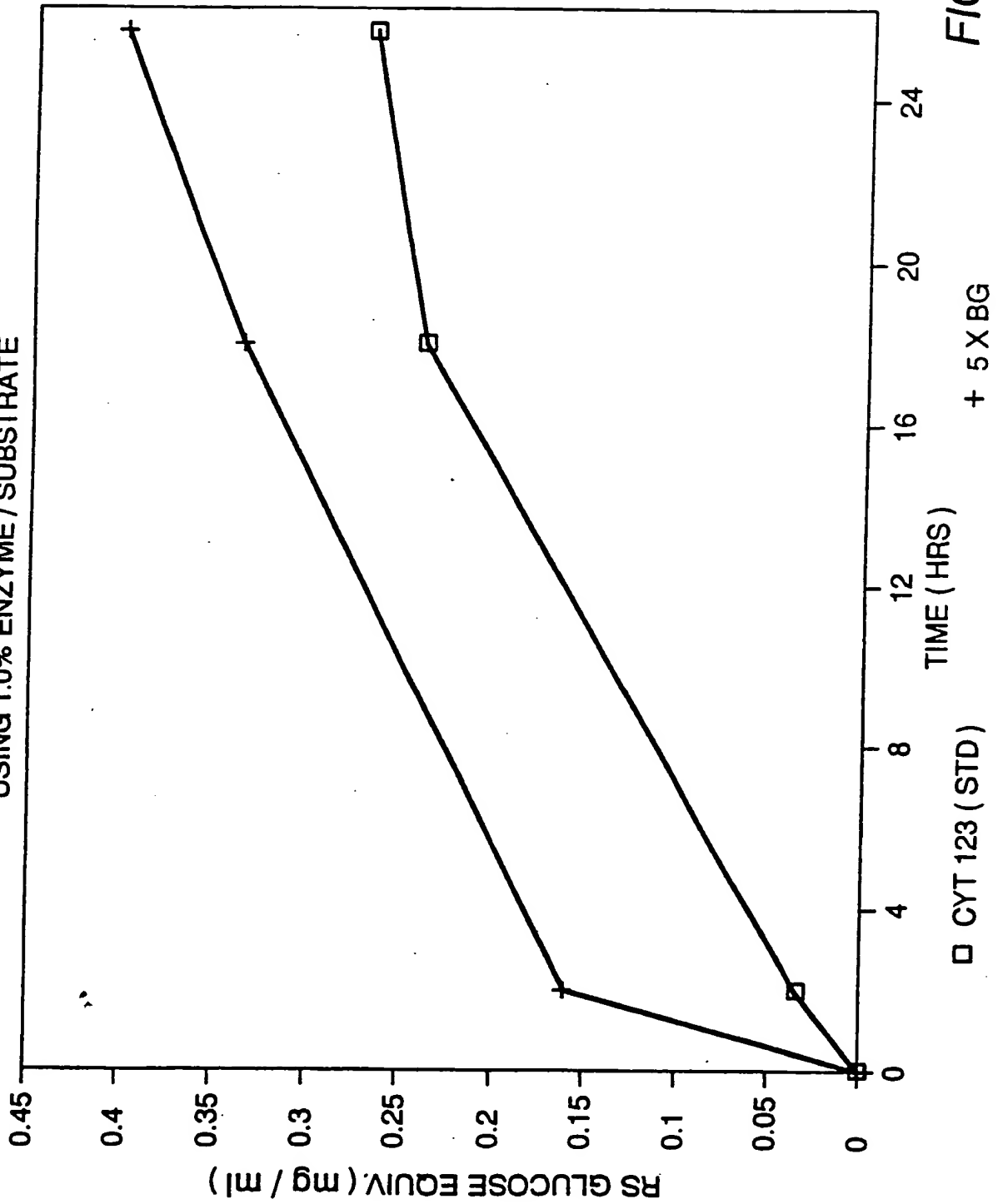
AVICEL HYDROLYSIS  
USING 1.0% ENZYME / SUBSTRATE

FIG. 7

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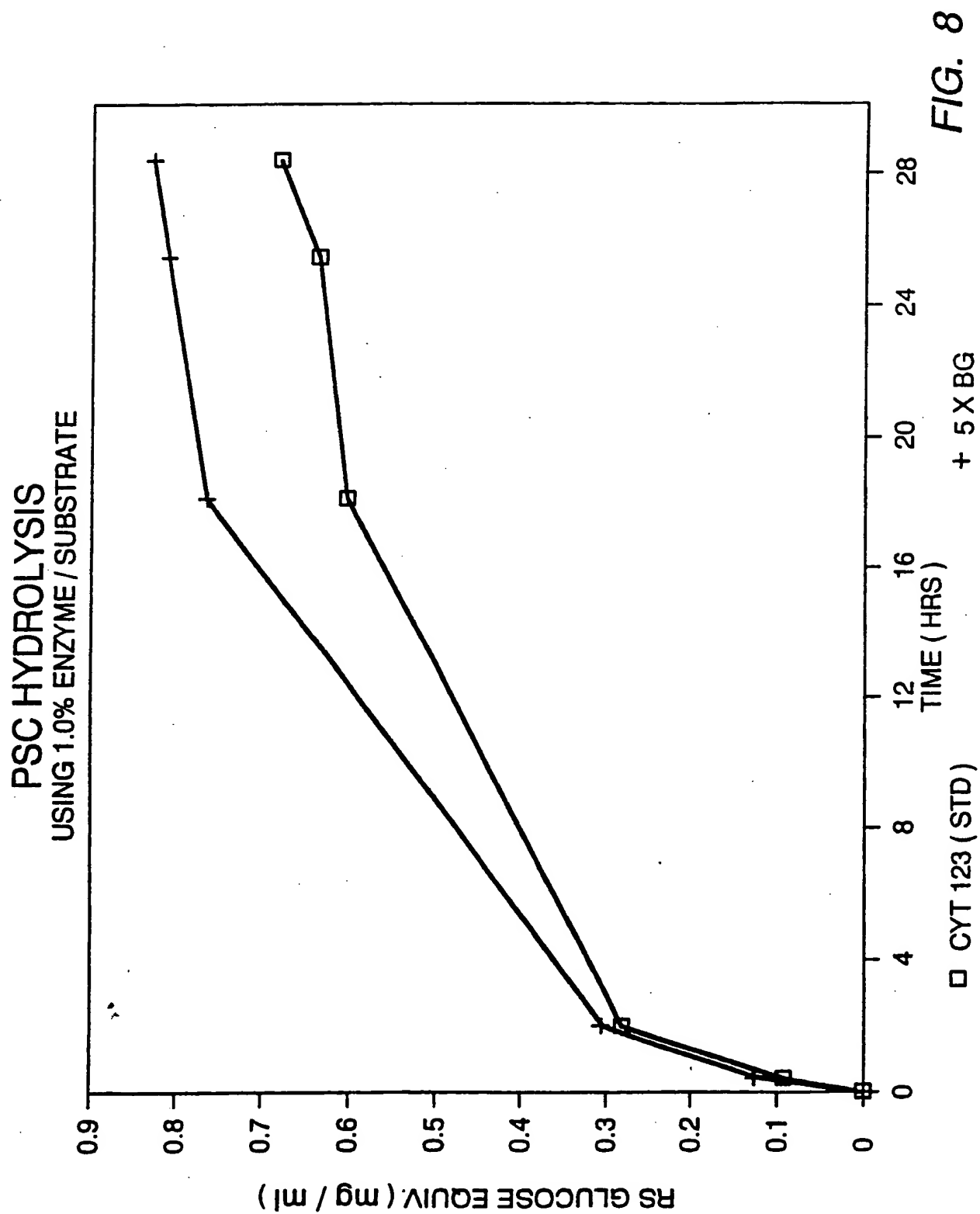


FIG. 8

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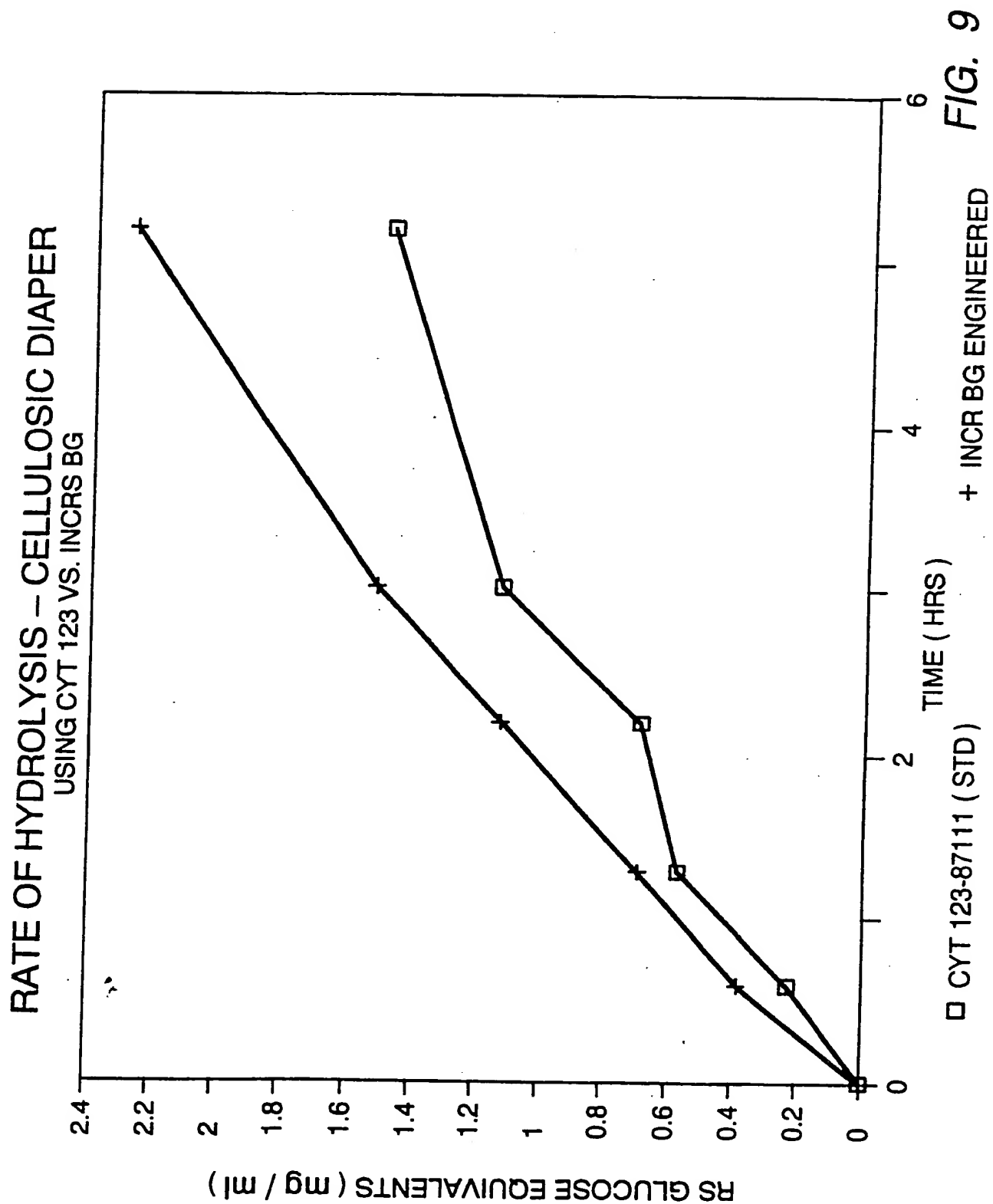
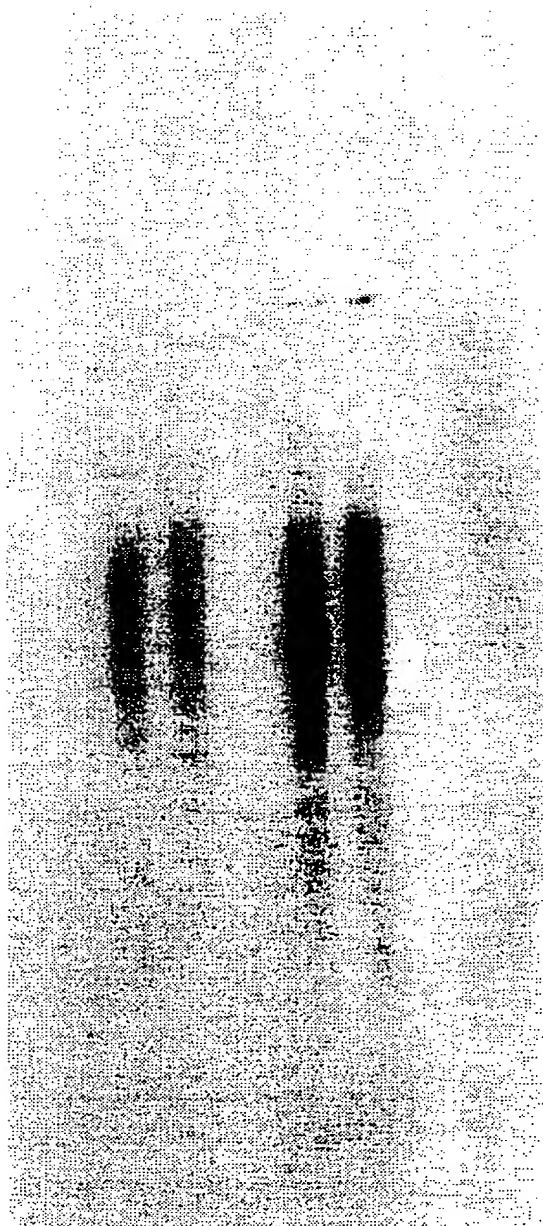


FIG. 9

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EcoRI  
HindIII

EcoRI  
HindIII



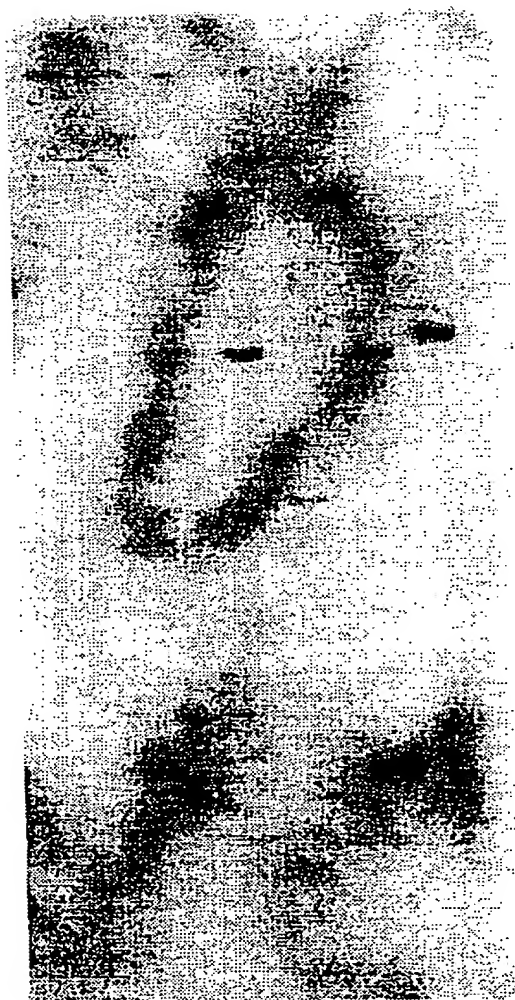
A. nidulans      N. crassa

FIG. 10A  
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BamHI  
EcoRI  
HindIII  
PstI



-14.1 Kb

-7.2

-6.3

-4.8

-4.3

-3.6

*Humicula grisea*

FIG. 10B

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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09285

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): C12P 21/00; C12N 1/14, 9/58, 15/80 US CL : Please See Attached Sheet.		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	435/69.1, 183, 223, 254, 263, 277, 320.1; 252/9.6, 287.1; 530/371, 412	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
APS, BIOSIS, CAS		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>*</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
A	Proceedings of the National Science Council Republic of China, Part B life Science, Vol. 12, No. 2, issued 1988, C.H. Wang et al., "An efficient approach for the preparation of fungal protoplasts: protoplast formation and regeneration in <u>Trichoderma kiningii</u> , pages 69-76. See abstract no. 6960172.	1-10, 13, 17-28, 32-43, 61-64
Y	Hoppe-Seyler's Zeitschrift fuer Physiologische Chemie, Vol. 355, No. 4, issued 1974, E. Bause, "Isolation and amino acid sequence of a hexa deca peptide from the active site of beta-glucosidase A-3 from <u>Aspergillus wentii</u> ", pages 438-442. See abstract no. 1585209.	1-10, 17-28, 32-43, 61-64
Y	Molecular General Genetics, Vol. 217, issued May 1989, W.L. Staudenbauer, "Nucleotide sequence of the <u>Clostridium thermocellum</u> bglB gene encoding thermostable beta-glucosidase B: homology to fungal beta-glucosidase", pages 70-76. See entire document.	1-10, 17-28, 32-43, 61-64
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents:<sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>	Date of Mailing of this International Search Report <sup>2</sup>	
21 MARCH 1992	23 APR 1992	
International Searching Authority <sup>1</sup>	Signature of Authorized Officer <sup>20</sup>	
ISA/US	John Leguyader	



## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Journal of Applied Biochemistry, Vol. 6, issued 1984, P. Bhikhabhai et al., "Isolation fo cellulolytic enzymes from <u>Trichoderma reesei</u> ", pages 336-345, see abstract no. 05644951.	11-12, 14-16, 29-31, 44-60, 65
A	<u>Trichoderma reesei</u> Cellulases, issued 1990, H. Merivuori et al., "Effects of alcohol and temperature on secretion by <u>Trichoderma</u> : cellulase complex production by <u>Trichoderma reesei</u> ; influenza A virus hemagglutin gene cloning and expression", pages 105-114, see abstract no. 91-09562.	1-10, 13, 17-28, 32-43, 61-64

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category <sup>16</sup>	Citation of Document <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
A	Bio/Technology, Vol. 9, issued June 1991, C.C. Barnett, "Cloning and amplification of the gene encoding and extracellular beta-glucosidase from <u>Trichoderma reesei</u> : evidence for improved rates of saccharification of cellulosic substrates: DNA sequence", pages 562-566.	1-10, 13, 17-28, 32-43, 61-64
Y	Protein Engineering, Vol. 3, No. 4, issued 1990, R.C. Miller, Jr. et al., "Enzyme mobilization or purification using the cellulose-binding domains of the two bacterial cellulases -- <u>Cellulomonas fimi</u> cellobiohydrolase and cellulase cellulose-binding domain, <u>Aspergillus</u> sp. beta-glucosidase, construction", page 379. See abstract no. 90-06574.	1-13, 17-28, 32-43, 61-64
A	Critical Rev. Biotechnol., Vol. 9, Vol. 2, issued 1989, V.S. Bisaria et al., "Regulatory aspects of cellulase biosynthesis and secretion: beta-glucosidase protein secretion", pages 64-103. See abstract no. 90-04716.	1-10, 13, 17-28, 32-43, 61-64
X/Y	Molecular General Genetics, Vol. 194, issued 1984, M.E. Penttila et al., "Cloning of <u>Aspergillus niger</u> genes in yeast. Expression of the gene coding <u>Aspergillus</u> beta-glucosidase", pages 494-499. See entire document.	1-10, 13, 17-28, 32-43, 61-64
Y	Current Genetics, Vol. 12, issued 1987, A. Raynal et al., "Sequence and transcription of the beta-glucosidase gene of <u>Kluyvermyces fragilis</u> in <u>Saccharomyces cerevisiae</u> ", pages 175-184. See entire document.	1-10, 13, 17-28, 32-43, 61-64
Y	Journal of Applied Bacteriology, Vol. 61, issued 1986, M.K. Kalra et al., "Partial purification, characterization and regulation of cellulolytic enzymes from <u>Trichoderma longibrachiatum</u> ", pages 73-80. See entire document.	11-12, 14-16, 29-31, 44-60, 65
Y	Proceedings of the National Academy of Sciences U.S.A., Vol. 82, issued 1985, M.M. Yelton et al., "A cosmid for selecting genes by complementation in <u>Aspergillus nidulans</u> : selection of the developmentally regulated <u>YA</u> locus", pages 834-838. See entire document.	1-10, 13, 17-28, 32-43, 61-64
A	Biochimica et Biophysica Acta, Vol. 626, issued 1980, Ernst Bause et al., "Isolation and structure of a tryptic glycopeptide from the active site of beta-glucosidase A3 from <u>Aspergillus wentii</u> ", pages 459-465.	1-65
Y	Journal of Bacteriology, Vol. 170, No. 1, issued January 1988, W.W. Wakarchuk et al., "Structure and transcription analysis of the gene encoding a cellobiose from <u>Agrobacterium</u> sp. strain ATCC 21400", pages 301-307. See entire document.	1-10, 13, 17-28, 32-43, 61-64
Y	Current Genetics, Vol. 12, issued 1987, M.E. Penttila et al., "Construction of brewer's yeast secreting fungal endo-beta-glucanase", pages 413-420. See entire document.	1-10, 13, 17-28, 32-43, 61-64
A	FEMS Microbiology Letters, Vol. 30, issued 1985, N.A. Sahasrabudhe et al., "Genome complexity of a powerful cellulolytic fungus, <u>Penicillium funiculosum</u> ", pages 295-300.	1-65

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	FEBS Congress 16th Meeting, Part C, issued 1985, J. Knowles et al., "The cloning of fungal cellulase genes and their expression in yeast: cellobiohydrolase and beta-glucosidase gene expression in transformed <u>Saccharomyces cerevisiae</u> ", pages 43-49. See abstract no. 86-06125.	1-10, 13, 17-28, 32-43, 61-64
Y	Biotechnol. Adv., Vol. 7, No. 3, issued 1989. B.R. Glick et al., "Isolation, characterization and manipulation of cellulase genes: cellobiohydrolase and beta-glucosidase genes cloning and expression; cellulase complex; a review", pages 361-386. See abstract no. 90-02275.	1-10, 13, 17-28, 32-43, 61-64
Y	DD, A, 263,571 (Humbolt Univ., Berlin) 07 August 1985. See abstract no. 86-01987.	1-10, 13, 17-28, 32-43, 61-64
Y	Abstr. Pap. Am. Chem. Soc., 194 Meeting, issued 1987, M. Gritzali, "Enzymes of the cellulase system of <u>Trichoderma</u> : an overview -- cellulase complex characterization". See abstract no. 88-04459.	11-12, 14-16, 29-31, 44-60, 65
Y	FEMB Symp., Vol. 43, issued 1988, H. Durand et al., "Classical and molecular genetics applied to <u>Trichoderma reesei</u> fro the selection of improved cellulolytic industrial strains --beta--glucosidase and cellulase activity; beta-D-fructofuranosidase gene cloning and expression in <u>Trichoderma reesei</u> ; cellulose degradation". See abstract no. 89-11621.	1-10, 13, 17-28, 32-43, 61-64
Y	Abstr. Pap. Am. Chem. Soc., 195 Meeting, issued 1988, C. Barnett et al., "Expression of <u>Trichoderma reesei</u> exo-cellobiohydrolase II genes in <u>Aspergillus awamori</u> : a heterologous expression system to study structure-function relationships --enzyme engineering applications". See abstract no. 88-08174.	1-10, 13, 17-28, 32-43, 61-64
Y	FEMS Symp., Vol. 43, issued 1988, J. Knowles et al., "The use of gene technology to investigate fungal cellulolytic enzymes -- <u>Trichoderma reesei</u> cellulase complex gene cloning and expression in <u>Saccharomyces cerevisiae</u> ". See abstract no. 89-11622.	1-65
Y	World Biotech. Rep., Vol. 2, issued 1984, S.P. Shoemaker et al., "The cellulase system of <u>Trichoderma reesei</u> : <u>Trichoderma</u> strain improvement and expression of <u>Trichoderma</u> cellulase in yeast -- in ethanol production", pages 593-600. See abstract no. 85-02556.	1-65
A	Biochem. Soc. Trans., Vol. 13, No. 2, issued 1985, K. Merivuori et al., "Regulation of cellulase biosynthesis and secretion in fungi -- <u>Trichoderma reesei</u> ", pages 411-414. See abstract no. 85-11852.	1-65
A	Abstr. Pap. Am. Chem. Soc., 202 Meeting, issued 1991, T. Fowler, "Developments in recombinant beta-glucosidase of <u>Trichoderma reesei</u> -- cellulase complex recombinant beta-glucosidase production". See abstract no. 91-14368.	1-10, 13, 17-28, 32-43, 61-64
A	US,A, 4,892,819, (Carr et al.) 09 January 1990. See entire document.	1-10, 13, 17-28, 32-43, 61-

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	US,A. 4,935,349, (McKnight et al.) 19 June 1990. See entire document.	64 1-10, 13, 17-28, 32-43, 61-64
Y	US,A. 4,275,163, (Gallo) 23 June 1981. See entire document.	1-65
Y	US,A. 4,885,252, (Ingolia et al.) 05 December 1989. See entire document.	1-10, 13, 17-28, 32-43, 61-64
Y	US,A. 4,472,504 (Gallo) 18 September 1984. See entire document.	1-65
X	WO,A. 9117244 (Woldike et al.) 14 November 1991, see abstract no. 008849748.	1-5
Y	Biotechnol., Vol. 7, No. 9, issued 1989, Ong et al., "The cellulose-binding domains of cellulases: tools for biotechnology-application in protein or fusion protein purification by affinity chromatography and in enzyme immobilization, a review", pages 239-243. See abstract no. 89-12873.	1-5
Y	Aust. J. Biotechnol., Vol. 3, No. 1, issued 1989, P.A.D. Rickard et al., "Kinetic properties and contribution to cellulose saccharification of a cloned <u>Pseudomonas</u> beat-glucosidase: enzyme cloning using plasmid pND71 and use of recombinant product in association with <u>Trichoderma reesei</u> cellulase", pages 43-49. See abstract no. 90-11472.	1-10, 13, 17-28, 32-43, 61-64

**FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS**  
(Not for publication)

**I. CLASSIFICATION OF SUBJECT MATTER:**  
US CL :

435/69.1, 183, 223, 254, 263, 277, 320.1; 252/8.6, 297.1; 530/371, 412

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